EXPLOITING TRADITIONAL CHINESE MEDICINE FOR POTENTIAL ANTI-MICROBIAL DRUG LEADS

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Abstract

Over the past few years, natural products have already become increasingly important in anti-infectious drug research. This is especially the case as innovations in omics' technologies, chemical biology and genetics have facilitated the discovery of new drugs and their targets. Natural products have a privileged role in the drug discovery due to their intrinsic cell permeability, structural diversity, rich functionality and stereochemistry. They also provide unique scaffolds for further drug optimization towards increased potency and selectivity. China has been using herbs in medicine for centuries and these are now attracting global interest. In response to the challenge of increased anti-microbial resistance our group established a China-UK collaborative team to explore the potential of Traditional Chinese Medicines (TCM) to reveal new drug leads.

This project focuses on 19 traditional Chinese herbs which were screened for a bioactives using a well-established analysis pipeline. All the plants were provided from China and underwent authentication using DNA barcoding and bioactivity screening of the extracts, mainly focusing on anti-microbial activities. Based on *rbcl* based DNA barcoding data some samples were mis-identified in China whilst some samples of the same species exhibited differences in minimum inhibitory concentrations (MIC).

The initial focus was on an assay-based evaluation of isolated compounds from *Artemisia annua* against mycobacteria. *Artemisia annua* is native to temperate Asia, known have antimalarial, anti-inflammatory, antitumor and allelopathic activities. Natural products with relatively weak anti-mycobacterial activity with MIC of 250 μg/mL from *Artemisia annua* were found with. Several fractions, isolated using different chromatography techniques, showed very significant anti-tubercular activity against *M. smegmatis*. Purification, isolation and identification of two compounds (artemisinic acid and deoxyartemisinin) using various chromatography techniques and identification using high-resolution mass spectrometry and nuclear magnetic resonance (NMR) with the best activity have been performed along with toxicity assays and *Schistosoma mansoni* assays. The mode of action using computer-predicting docking with other known targets of *M. tuberculosis*. Based on the MIC pattern and docking algorithms it was predicted that artemisinic acid could probably target KasA protein of *Mycobacterium*.

Next the antimicrobial properties of herbs, *Dryopteris crassirhizoma* Nakai were identified as potential drug lead against Methicillin-resistant *Staphylococcus aureus* (MRSA). Bioactive phloroglucinols from *D. crassirhizoma* were isolated, purified, and identified using a range of bioactivity screens coupled with chromatographic and metabolomics approaches. The antimicrobial properties of the extracts and fractions yielded MIC ranging from 3.25-50 μg/mL. The potential compounds responsible for MIC were tentatively identified through metabolite fingerprinting using flow infusion electrospray high-resolution mass spectrometry (FIE-HRMS). Additionally, the mode of action of the selected fractions was suggested using non-targeted metabolomics analysis with other known antibiotics and docking with known targets. Metabolomics data shows that the anti-MRSA activity was linked to the altered carbohydrate metabolism that could reflect the inhibition of growth of the cells.

This study requires further validation for the compounds from *D. crassirhizoma* using genomics, proteomic, and lipidomic analyses and *in silico* studies. Overall, our study can help find different naturally available compound and help in the identification of targets to fight different diseases including the drug resistant disease.

Acknowledgement

First and foremost, I would like to thank to my supervisors Professor Luis Mur and Dr. Manfred Beckmann for their tireless assistance, advice and coordination throughout this successful project. Thank you for this opportunity to learn and grow under your guidance.

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Preface

This thesis focuses on revisiting traditional medicine and investigate their chemical entity in the search of a new drug and new mechanisms of action against tuberculosis and MRSA. **Chapter 1** briefly reviews the causes of antimicrobial resistance, the importance of natural products and known natural products, as source of alternative, drugs; Traditional Chinese medicine in comparison with the western medicine and how it represents an important corpus of "folk-medicine" are presented. Finally, the aims and objectives of this thesis are described in this chapter. **Chapter 2** introduces the material and methods commonly utilised throughout the thesis. With the increase in consumer demand **Chapter 3** introduces different methods used to authenticating traditional Chinese medicine and derives an analysis pipeline which is used throughout the thesis. **Chapter 4** focuses on *Artemisia annnua* and screens for antimycobacterial activity. Here bioassays- guided the purification of chemical with *in vitro* anti-microbial activity. In this chapter, computational molecular docking is used to predict the interaction sites. **Chapter 5** introduces to *Dryopteris crassirhizoma* and focuses on its anti-MRSA activity. It also employs a similar bioassay guided purification as used in chapter 4. Also, in this chapter metabolomics technology is applied to rapidly elucidate potential bioactives in a complex biochemical mix. In **Chapter 6** metabolomics is used to target likely bioactives with anti-MRSA functions in a fraction from *D. crassirhizoma*. **Chapter 7** integrates and discusses all the results obtained in the previous chapters, providing a critical global overview and future perspectives on drug and target discovery of natural products. **Chapter 8** presents the publications, posters and oral communications related to this thesis. A subchapter of non-thesis related publications is also described.

Antimicrobial resistance and Natural products

1.1. ANTIMICROBIAL RESISTANCE (AMR)

Introduction

The continual battle between human and infection causing microorganisms turned in favour of humans in the middle of 20th century when penicillin became available to use. Equally, the anti-microbial importance of sulphonamides need to he acknowledged. As early, as 1935 (penicillin was not widely used until the 1940s), the first sulphonamide, prontosil was synthesized against Streptococcus infections by Gerhard Domagk (Cohen & Swerdlik, 2009). However, this success was short lived as with time the pathogens countered back with various forms of resistance. The struggle to gain the upper hand against infection is still on. Scientists working on new antimicrobial agents are struggling as organisms are evolving cleaver mechanisms of resistance (Tenover, 2006). The period after 1980 is defined as an "antibiotic discovery void" as no successful antibiotic discoveries are made also no new class of antibiotics has been discovered to treat Gram-negative bacteria since 1962 (Silver, 2011) (Figure 1.1)

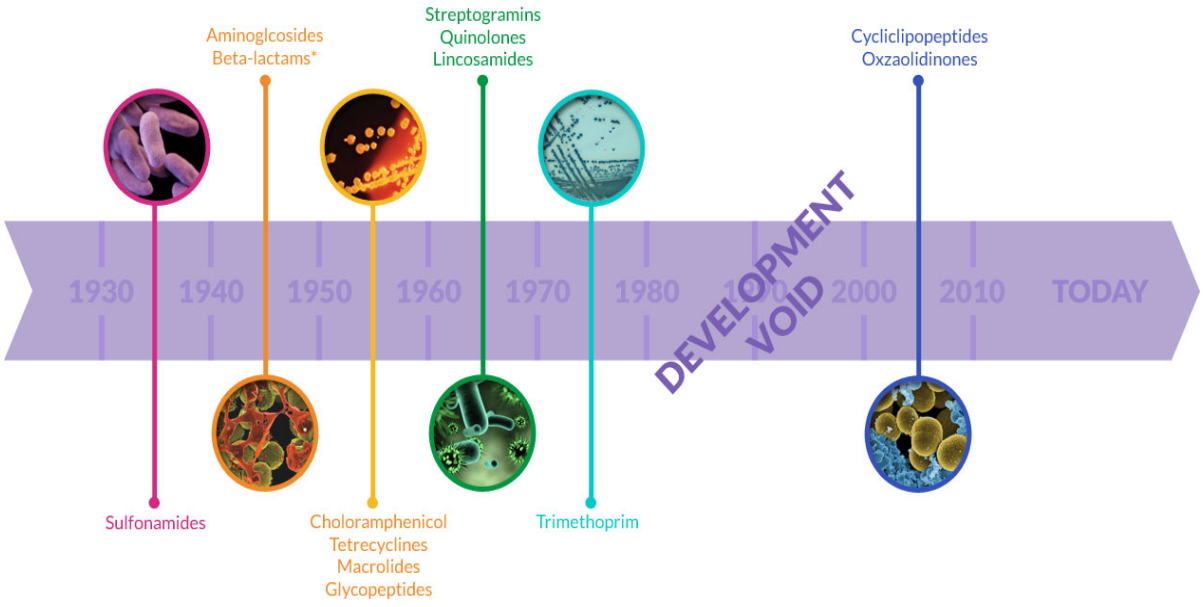


Figure 1.1 Discovery of new classes of antibacterial drugs has stalled (1930s to 2000s).

Source: World Health

Teixobactin, a new antibiotic after nearly 30 years have been discovered to be active against a broad range of organisms like *Streptococcus pneumoniae*, *Staphyloccocus aureus* (MRSA) and *Mycobacterium tuberculosis* without any noticeable resistance (Ling et al., 2015).

Alexander Fleming's Nobel Prize acceptance speech stated, "it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill the and the same thing has occasionally happened in the body. There is the danger that the ignorant man may easily under-dose himself and, by exposing his microbes to non-lethal quantities of the drug, make them resistant" (Fleming, 1945). Currently, almost all strains in UK and USA are resistant to penicillin and more than 50% are resistance to methicillin (Huttner et al., 2013). Antimicrobial resistance (AMR) is recognized as one of the greatest threats to human health worldwide by organizations such as the US Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO). The period between 2014 to 2016 has observed an estimation of ~1 million deaths and suspects ~300 million deaths by the year 2050 due to AMR (O'neill, 2016) (Figure 1.2) Resistance among gram-positive organisms is very

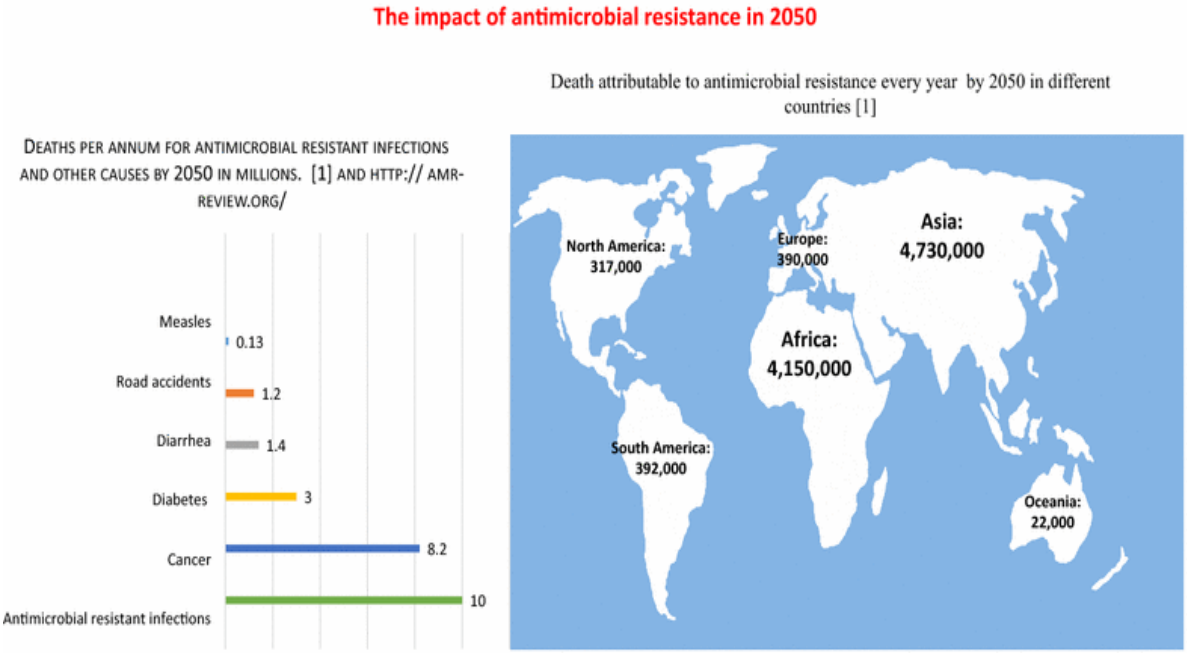


Figure 1.2: The impact of antimicrobial resistance in 2050.

(Bassetti et al., 2017)

common while in contrast the resistance in gram-negatives is relatively rarer but in both groups of bacterial it is an urgent concern.

Cause of resistance

The main reason of AMR is generally associated to the selective pressure caused by the improper use, overuse, or misuse of antimicrobials in humans and animals (Who_Food_2011, 2011). Individually a microbe is limited in its ability to cope with diverse environmental factors, but when in communities these microbes are extremely adaptable. The main reason for this adaptability is the rapid generation time, which help them, have a new evolved species without losing the adaptive genes. Antimicrobials are used to block the growth of microorganisms, so under this treatment microbes that can overcome this selective pressure and survive to become predominant in an evolved microbial community. Persistent use of antimicrobials will drive the selection pressure causing them to evolve faster.

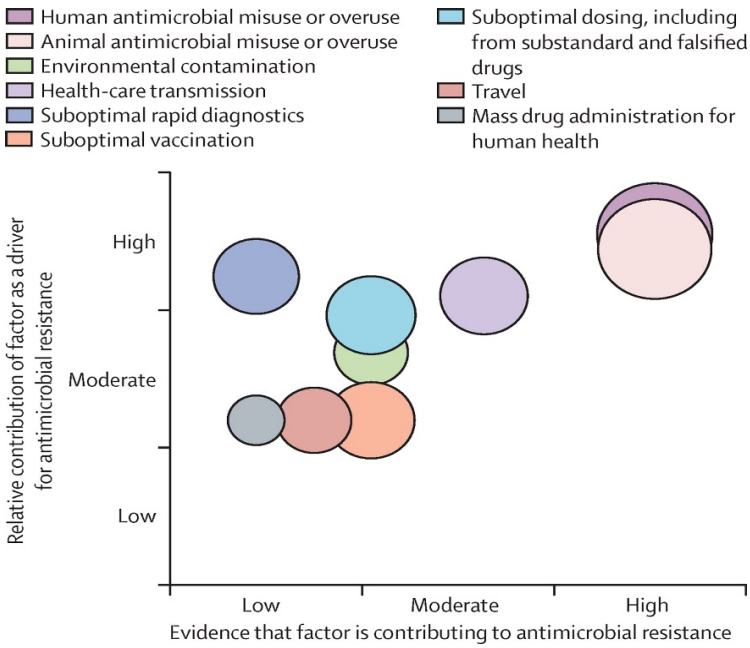


Figure 1.3: An infographic to show the considered potential contribution of each factor as a driver for antimicrobial resistance.

(Holmes et al., 2016)

Three major factors determine that cause resistance: (1) the increasing frequency of AMR phenotypes among microbes due to the widespread use of antimicrobials; (2) the large and globally connected human population allowing bacterial spread and (3) the extensive and often unnecessary use of antimicrobials by humanity providing a strong selective pressure (Michael, Dominey-Howes, & Labbate, 2014) (Figure 1.3).

Antimicrobial resistant bacteria is naturally present in environment including Antarctica as they coexists with non-resistant species for stability (Martinez, 2009). Further, bacterial species that would normally be considered as gut microflora are found in the environment, drinking

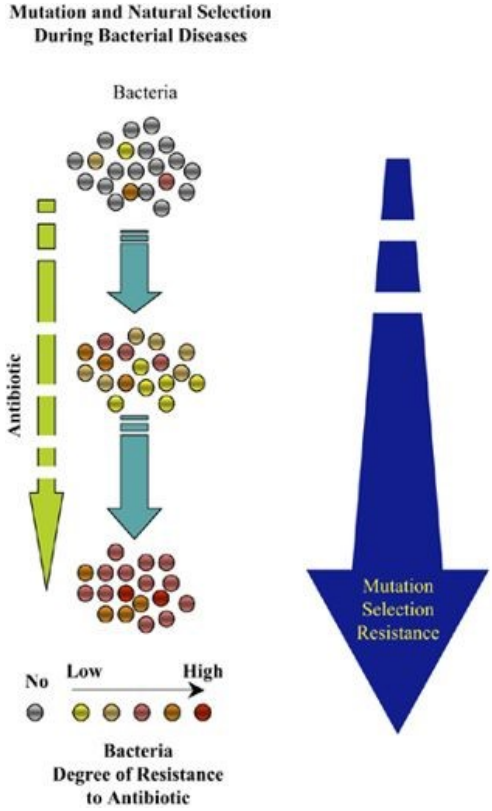


Figure 1. 4: Mutation selection resistance.

The population of bacteria, before exposure to an antibiotic, is composed of genetically variable bacteria. Upon repeated short exposure to antibiotic, natural selection takes place with survival of resistant bacteria. (Lagasse, 2008)

water, soil, food, and food products (Rubin, Ekanayake, & Fernando, 2014) (Walsh, Weeks, Livermore, & Toleman, 2011). This represents a continuum for environmental AMR to enter humans. However, it is selective pressure exerted within human use of antibiotics that is the

main driver for AMR. It is suggested that 50 % antimicrobials prescribed to patients are unnecessary, so that their excessive use of is selecting for resistant organisms in patients (Frieden, 2013). As the human body is a complex system, several factor influence the derivation of resistant strains including pathogen-drug interactions, pathogen-host interactions, mutation rates of the pathogen, emergence of successful antimicrobial-resistant clones, the transmission rates of pathogens between human beings, animals, and the environment, cross-resistance, and selection of co-resistance to unrelated drugs (Figure 1. 4) (Holmes et al., 2016). However, several other external factors lead to resistance such as the use of antibiotics in food products, sanitization. Other social factors such as population densities and the efficacy of the health care system are also important (Turnidge & Christiansen, 2005).

Handling antimicrobial resistance

Due to the urgency to deal with this antimicrobial crisis healthcare bodies such as CDC (Frieden, 2013) and the WHO (World Health Organisation, 2015) have provided a series of recommendations to manage the crisis. To decrease the rise and spread of MDR, cooperative efforts are required to control the factors responsible for resistance in particular, the use of antimicrobials and reducing spread of resistant microorganisms. Several antimicrobial conservation (AC) programs (traditionally known as antibiotic stewardship programs) have been suggested to optimize antibiotic prescription practices, however the program has had limited reach worldwide. Among several AC initiatives, the reduced prescription of floroquinone and cephalosporin resulted to have resulted in a 70 % reduction in AMR *Clostridium difficile* infections (CDI) in UK over the past five years (Huttner et al., 2013).

Besides direct human consumption, the largest volumes of antimicrobials is used in livestock production which become a source of AMR. To support antimicrobial stewardship, the WHO has promoted an integrated surveillance of consumption and resistance in both the human and

animal sectors (WHO, 2017) and have listed the "critically important" antimicrobials to be used use in development and implementation of risk management strategies in livestock production (Figure 1.5). Withdrawing the use of a third-generation cephalosporin (ceftiofur) in Canada with a resulting reduction in resistance rates in *Salmonella enterica* serovar Heidelberg is an example of the success of such a strategy (Dutil et al., 2010; Willemsen et al., 2015).

Figure 1.5: Different ways to handle antimicrobial resistance.

1.2. NATURAL PRODUCTS

Natural environment is frequently identified as a rich source of unique chemical diversity for pharmaceutical lead-compound discovery. According to an estimate, about 50 % of the medications validated from 1981-2010 have natural origins. (Mushtaq, Abbasi, Uzair, & Abbasi, 2018)

The investigation of natural products as source of novel human therapeutics reached its peak in the Western pharmaceutical industry in the period 1970–1980, which resulted in a pharmaceutical landscape heavily influenced by non-synthetic molecules. Despite this success, pharmaceutical research into natural products has experienced a slow decline. However, the emergence of multidrug-resistant microbes has increased the urge to search for new therapeutic lead compounds. Major advantages of a natural product in comparison to synthetic entity are that they exhibit tremendous chemical and structural. Also, they are more prone to evolutionary pressure compared to a synthetic molecule. Natural products can be obtained from four main sources i.e., plants, animals, marine organisms and microorganisms

Microorganisms as a source of natural products

The discovery of penicillin from the filamentous fungus, *Penicillium notatum* in 1929 started the Golden Age of antibiotics with intensive research on nature as source of novel bioactives (Swann, 1992).There are about 25,000 species of basidiomycetes, of which about 500 are members of the Aphyllophorales. Approximately 75% of tested polypore fungi have shown strong antimicrobial antiviral, cytotoxic, antineoplastic, cardiovascular, anti-inflammatory, immune-stimulating and anticancer activities (Stamets, 2002; Zjawiony, 2004).

Figure 1.6 shows some examples of microbe isolated natural product such as vancomycin (**1**) that was isolated from *Amycolatopsis orientalis* in 1953 and has been proven to be active against a broad range of organisms and was approved by FDA (Federal Drug Agency, USA) in 1958. Erythromycin (**2**), originated from *Saccharopolyspora erythraea* and is also a broad spectrum antibiotic against gram positive bacterial species (Butler, 2004; Dewick, 2002). Ganoderic acid β (**3**) was isolated from the fruiting bodies and spores of *Ganoderma lucidum*, and displayed significant anti-HIV-1 protease activity with an IC50 value of 20 μM (Min,

Nakamura, Miyashiro, Bae, & Hattori, 1998). Doxorubicin (**4**) (Adriamycin®), isolated from *Streptomyces peucetius* is used to treat acute leukaemia, soft tissue and bone sarcomas, lung

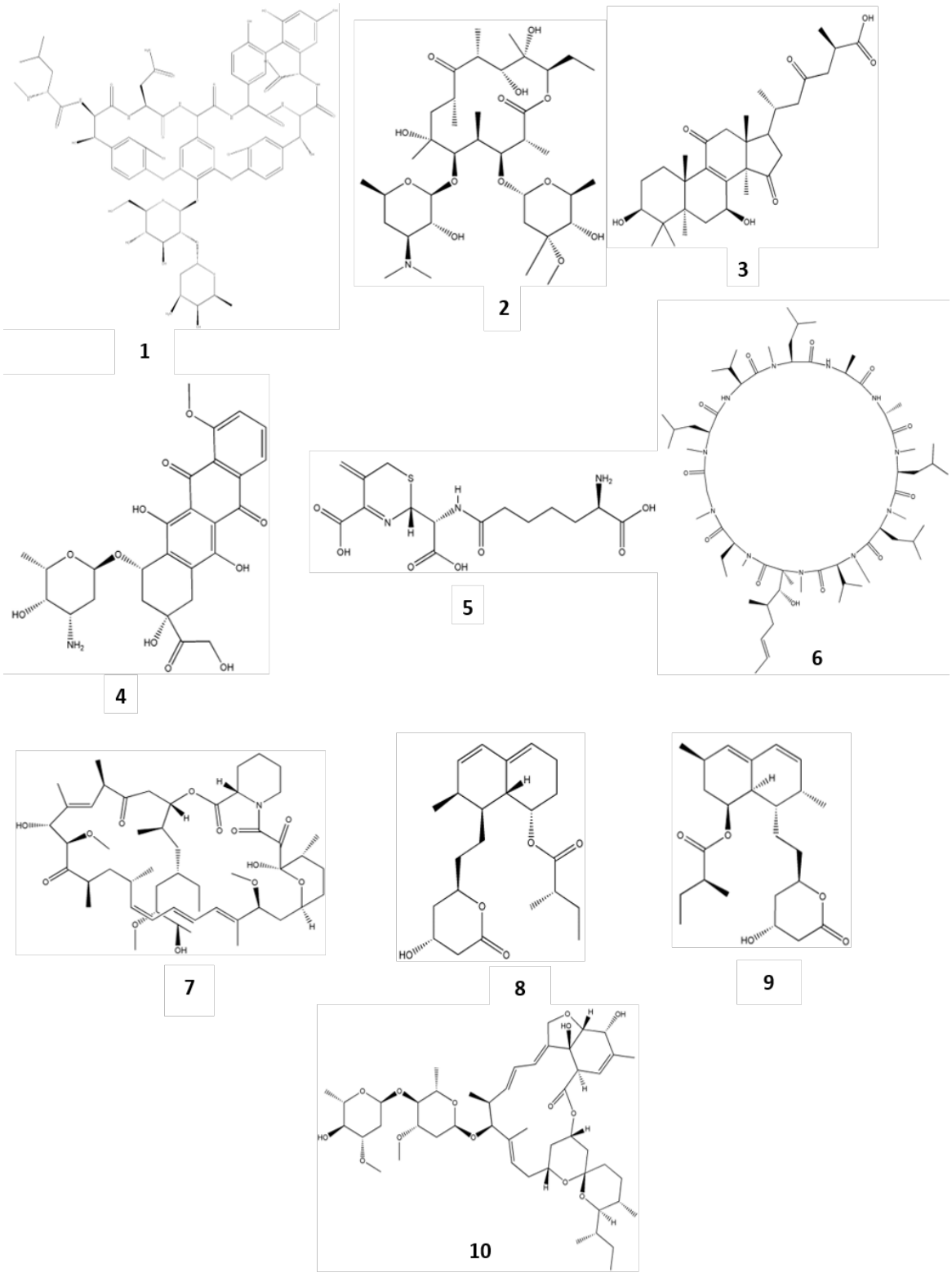


Figure 1.6: Some examples of microbe-derived natural products.

1: vancomycin; 2: erythromycin; 3: ganoderic acid β; 4: doxorubicin; 5: cephalosporins; 6: cyclosporine; 7: rapamycin; 8: mevastatin; 9: lovastatin; 10: ivermectins.

cancer, thyroid cancer and both Hodgkin's and non-Hodgkin's lymphomas (Butler, 2004; Dewick, 2002). Amongst a plethora of possible other example it is possible to include antibacterial agents such as cephalosporins (**5**) from *Cephalosporium cryptosporium*; aminoglycosides, tetracyclines , and other polyketides of many structural types from the *Actinomycetales* sp.; immunosuppressive agents like cyclosporine (**6**), and rapamycin (**7**) from *Streptomyces* species; cholesterol-lowering agents such as mevastatin (**8**) and lovastatin (**9**) from *Penicillium* species and anthelmintics, antiparasitic drugs, such as the ivermectins (**10**) from *Streptomyces* species (Goodman & Ro, 1995).

Plants as a source of natural products

The earliest records of the uses of a natural products is from 2600 B.C which describes the use of oils from *Cupressus sempervirens* (Cypress) and Commiphora species (myrrh) *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh), and *Papaver somniferum* (poppy juice) to treat cough, colds, parasitic infections and inflammations (Cragg & Newman, 2005). An Egyptian pharmaceutical record of 2900 B.C documents the use of 700 plant-based drugs. Likewise, the Chinese Materia Medica from1100 B.C and the Indian Ayurveda system dates from about 1000 B.C provide extensive examples of the use of natural products in medicine. A similar comprehensive medical compendium is not seen in the western world until the work of Theophrastus (~300 B.C.) a Greek philosopher and natural scientist who described the medicinal qualities of herbs in the *History of Plants*.

Plants have been an important source of medicines for thousands of years. World Health Organization (WHO) states that 80% of people rely on plant-based medicine for their primary health care needs (Fabricant & Farnsworth, 2001). Beyond this, further investigation of medicinal plants has resulted in the isolation of many natural products which have been developed as front-line drugs. For example, paclitaxel (Taxol®) (**11**) widely used breast cancer

drug, was isolated from *Taxus brevifolia* (Pacific Yew) by the United States Department of Agriculture (USDA) in 1962 (Cragg, 1998) and obtained FDA approval for its clinical use in 1992 (Kaufman, Cseke, Warber, Brielmann, & Duke, 2010). As Taxol® (**11**) is present only at low quantities in its natural source, its large scale use required it chemical synthesis to be established (Nicolaou et al., 1995).

Figure 1.7 shows a wide range of plant derived examples such as Ingenol 3-*O*-angelate (**12**), a derivative of the polyhydroxy diterpenoid ingenol was isolated from the sap of *Euphorbia peplus* (known as "*petty spurge*" in England or "*radium weed*" in Australia), and is currently under clinical development by Peplin Biotech as a potential anti-tumour compound (Kedei et al., 2004) although more recently the European Medicines Agency (EMA) has highlighted an increased risk of skin cancer with this drug (Schmutz, 2020). 14-succinyl triptolide sodium salt (**13**) is a semisynthetic analogue of triptolide isolated from *Tripterygium wilfordii* used for autoimmune and inflammatory diseases in China (Fidler et al., 2003; Kiviharju, Lecane, Sellers, & Peehl, 2002). Combretastatin A-4 phosphate (**14**) a stilbene derivative from *Combretum caffrum,* an anti-angiogenic agent, is currently in Phase II clinical trials (Holwell et al., 2002; Newman & Cragg, 2005). Artemisinin (**15**), arteether (**16**) and its derivatives were isolated from *Artemisia annua* are known to have antimalarial properties. Artemisinin (**15**) as artemisin and arteether (**16**) as Artemotil are approved as antimalarial drugs while other derivatives are in various stages of clinical development (Cragg & Newman, 2005; Dewick, 2002). Potential analgesic agents Grandisines A (**17**) and B (**18**) were isolated from the leaves of the Australian rainforest tree, *Elaeocarpus grandis* (Carroll et al., 2005). Galantamine hydrobromide (**19**) an Amaryllidaceae alkaloid from the plant *Galanthus nivalis* is used for the treatment of Alzheimer's disease (Heinrich & Teoh, 2004; Howes, Perry, & Houghton, 2003). Apomorphine hydrochloride (**20**), a potent dopamine receptor agonist used to treat Parkinson's

disease, a derivative of morphine isolated from *Papaver somniferum* (Deleu, Hanssens, & Northway, 2004).

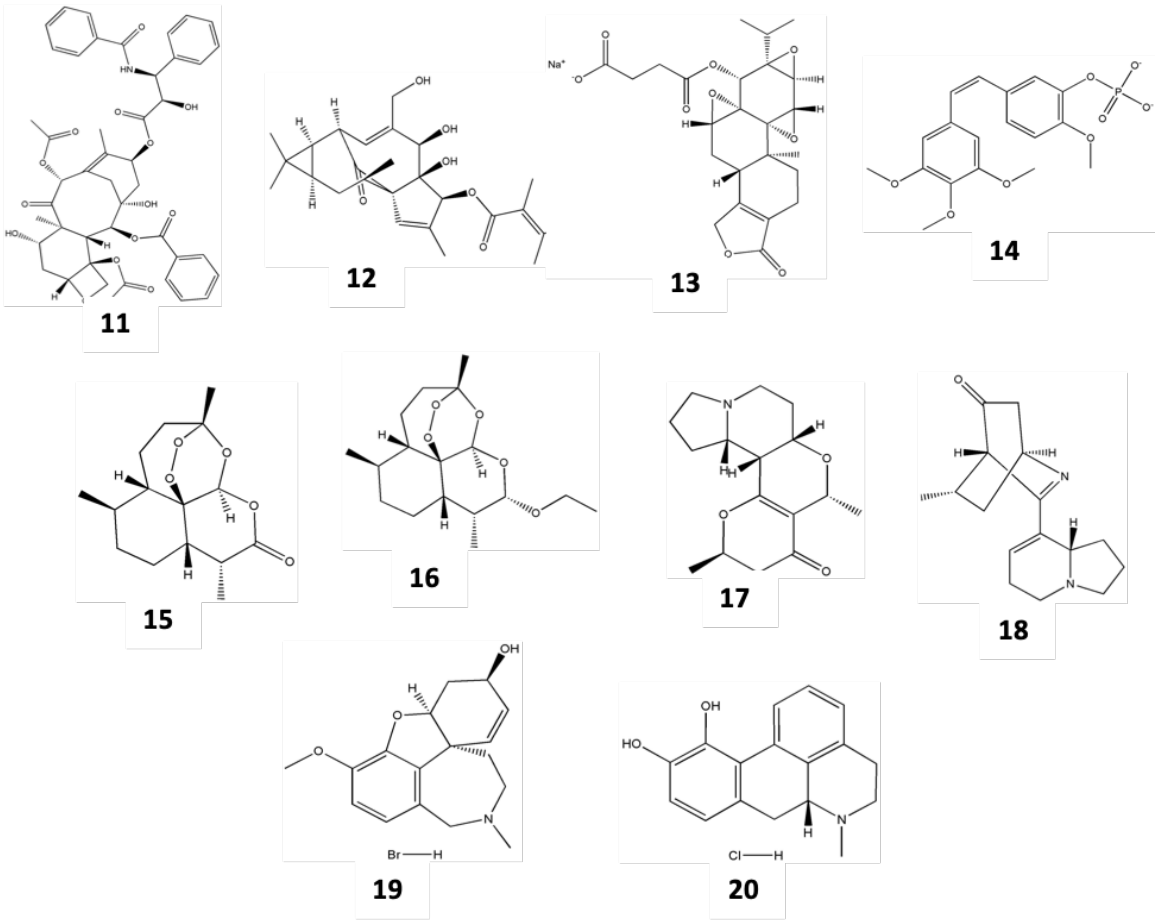


Figure 1.7: Some examples of Plant-driven natural products.

11: Taxol®; 12:ingenol 3-O-angelate; 13:14-succinyl triptolide sodium salt; 14:combretastatin A-4 phosphate; 15:artemisinin; 16:arteether; 17:grandisines A; 18: grandisines B; 19:galantamine hydrobromide; 20:apomorphine hydrochloride.

Marine organisms as a source of natural products

Our earth is covered by 70% of oceanic water, hence has enormous resources that are still being explored. Since the techniques to explore, the seabed is very recent, no historical evidence of traditional applications from marine sources have been found. In early 1950's C-nucleosides, spongouridine, and spongothymidine, was isolated from the Caribbean sponge, *Cryptotheca crypta*. These antiviral compounds were eventually used to synthesize cytosine arabinoside (Ara-C) clinically useful anticancer agent (McConnell, Longley, & Koehn, 1994). With this

discovery, there was an increased interest in exploring bioactive in marine environment. From past 40 years several studies proved that marine environment is a great source of bioactive but no compound has pass through clinical trials.

Most notable example is bryostatin 1 (**21**) anti-cancer agent, isolated from the bryozoan, *Bugula neritina* though has completed both phase I and phase II levels but only in combination with other cytotoxic drugs (Mayer et al., 2010). Dolastatin 10 (**22**), a linear depsipeptide another anticancer drug from *Dolabella auricularia* did manage to overcome Phase I clinical trials but failed in phase II trials due to lack of significant activity (Newman & Cragg, 2005). Plitidepsin (**23**) (Aplidin®, PharmaMa), a depsipeptide anti-cancer agent isolated from the Mediterranean tunicate *Aplidium albicans*, currently in phase II clinical trials (Mayer et al., 2010) (Urdiales, Morata, De Castro, & Sánchez-Jiménez, 1996). Ecteinascidin 743 (ET743;

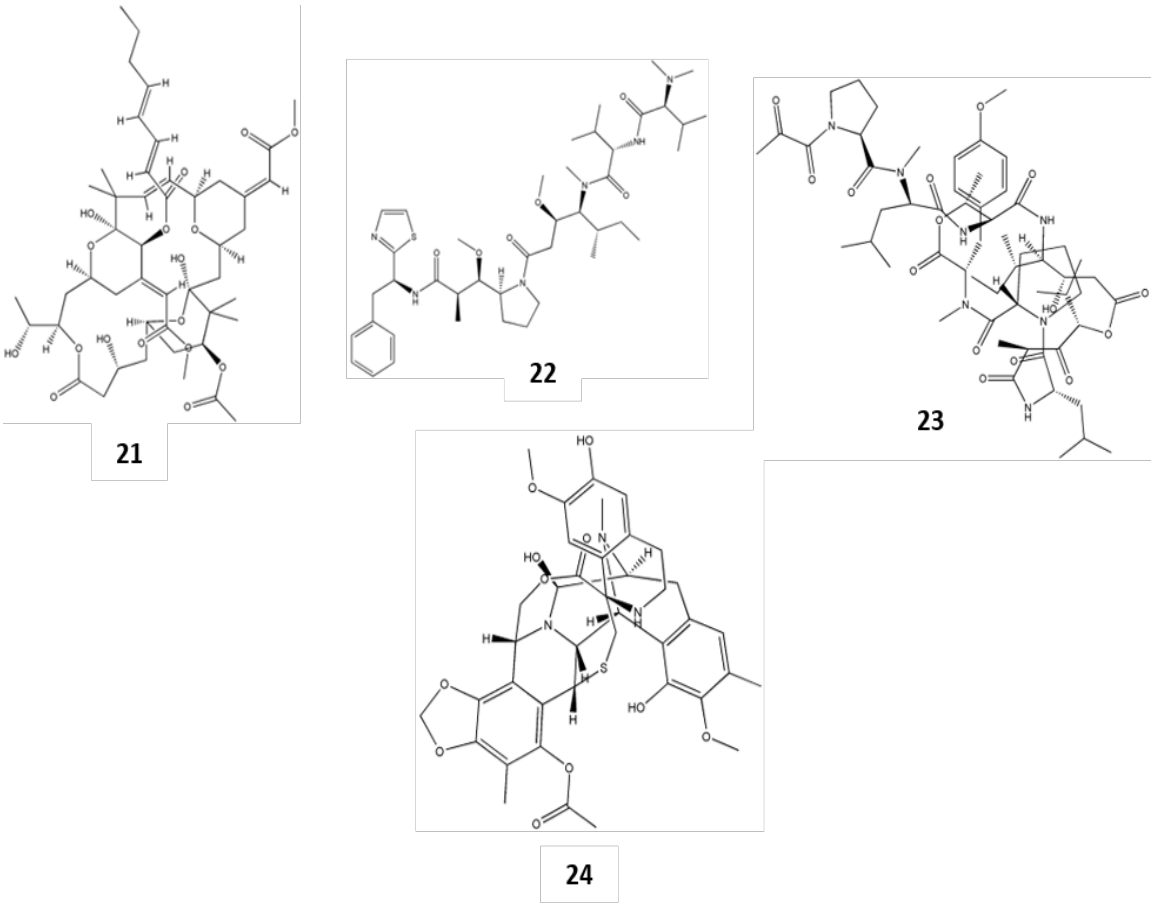


Figure 1.8: Some examples of Marine-driven natural products.

21: bryostatin 1; 22: dolastatin 10; 23: plitidepsin; 24:Yondelis™.

Yondelis™) (**24**), became the first marine anticancer drug to be approved in the European Union in 2007 (Figure 1.8)

Importance and limitations of plant natural products as antimicrobials

The plant kingdom includes a high number of species, producing highly diverse bioactive compounds. According to some estimates, only 6% of plant species have been systematically investigated for their pharmacological properties. This represents a missed opportunity as to date of 122 plant derived compounds in clinical use of which 80% have ethno-medical origins. Papaverine from *Papaver somniferum* (Figure 1. 9) is probably the best example of ethno medical information guiding drug discovery and development along with the antimalarial drugs, quinine and artemisinin (15) (Cragg & Newman, 2013). Although the isolation of quinine from the bark of *Cinchona* species was reported in 1820 (Buss, Cox, & Waigh, 2003) it was target as a result of its traditionally use by tribes in the Amazon region in treating fever. With the emergence of resistance to synthetically derived quinine drugs, *Artemisia annua* (Quinhaosu), long known in TCM, has gained prominence as a frontline drug for malaria (Miller & Su, 2011).

As plants used for medicinal purposes obtained from natural habitat, it is of extreme importance that the plants identified genetically, chemically besides morphological and anatomical

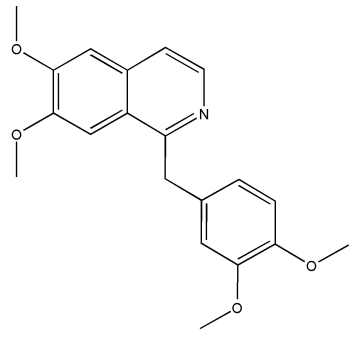


Figure 1. 9: Chemical structure of Papaverine.

identification. However, with climate change, there is a great risk of endangering the sources

of potential drugs from nature. This is not only through species loss, continuous environmental changes leads to biochemical changes in plants which could include the loss of key bioactives (Bucar, Wube, & Schmid, 2013; David, Wolfender, & Dias, 2015). It is vital to consider the collection of protected species in the right season and habitat. The United Nation's Convention on Biological Diversity (CBD) (CDB, 1992) by the international community in Rio de Janeiro, Brazil, aims at: (1) conserving the biodiversity; (2) sustainably using its genetic resources; and (3) sharing the benefits from their use in a fair and equitable manner. Although the CBD provided a framework for countries to regulate and define bioprospecting, the treaty left many open questions, particularly in the issue of access and benefit sharing, resulting in reduction of access to the plant material and thus decreased interest of pharmaceutical companies for plant based drug discoveries (Cragg & Newman, 2013; Kingston, 2011). To overcome this, the Nagoya Protocol (CDB, 2010) on access to genetic resources and the fair and equitable sharing of benefits arising from their utilization to the convention on biological diversity has been published in 2011 and has come into force in October 2014.

Besides the accessibility of the plant material, also its quality is of great importance. The chemical composition of a plant depends on various factors like species identity and harvest time, but also on soil composition, altitude, actual climate, processing, and storage conditions. Another aspect determining the chemical composition of the starting plant material is presence and absence of endophytic organisms, inhabiting the plant as the metabolites might alter due to the interaction of organisms (David et al., 2015).

Another important barrier are accessibility of the starting material and the low yield of bioactives. Larger quantity of bioactives required to characterise a potential pharmacological lead and is a frustrating drawback especially when initial investigations are promising. This low yield is especially problematic as the investigation of plant extracts using involves high-through put screening (HTS), then this is followed by the identification of bioactive

constituents. In general, HTS requires high reproducibility, accuracy, robustness, and reliable liquid handling systems. Test compounds should not decompose or precipitate, should not interfere with assay reagents nor show non-specific effects and natural products often fail to fulfil these requirements. Plant extracts are usually complex, viscous, tends to precipitate and sometime contains components like inorganic components, fluorescent components, organic components that non-specifically bind proteins, or have toxic constituents might interfere with assays (Coan, Ottl, & Klumpp, 2011; Henrich & Beutler, 2013; Johnson et al., 2011; Maes et al., 2012; Schmid, Sattler, Grabley, & Thiericke, 1999; Ying Tu et al., 2010). The technical demands linked to natural products characterisation leads to development costs that are much higher than the costs for small molecule drugs. As a result interest in natural product-based drug discovery has been declining (Beutler, 2009; David et al., 2015; Ortholand & Ganesan, 2004).

1.3. TRADITIONAL CHINESE MEDICINE

China is the second largest country based on area and this results in wide differences in topography, climate and soil types. As a result, it is considered to be the third most bio-diverse country in the world. China has over 32,000 species of vascular plants and 10,000 recorded species of Fungi. Out of such biodiversity, over 7000 species have medicinal uses. China has been using herbs for centuries as traditional Chinese medicine (TCM) and these are now attracting global interest as a source of natural products. However, the information regarding their use is often inaccessible to the wider scientific community. With the increased export of Chinese Medicines, global access to TCM has never been easier but there remain difficulties in targeting the most appropriate sources, and product quality assessments.

Western medicine was introduced into China in the sixteenth century, but it did not obtain any wide acceptance in that country until the nineteenth century. This was due to its competition with traditional Chinese medicine (TCM), widely used by the Chinese for centuries. Shen Nong is regarded as the father of Medicine (K. C. Wong & Wu, 1936). and founder of Chinese medicine. The "*Classic of Shen Nong's Materia Medica*" is the first book summarizing the properties and functions of over 300 herbs (P. Chen, Lu, & Lin, 1997). The *Yellow Emperor's Inner Canon* is the oldest work of Chinese medical theory (dated around the first century BC), and explains the relationship between humans and their environment as it impacts on the body, vitality, and pathology. Methods of diagnosis and therapeutic approaches ideas were developed based on this ancient philosophy (Boltz & Loewe, 1993; Harper, 2013; G.-D. Lu & Needham, 2012). The concept of Yin, Yang and Five Phases (Ellis & Wiseman, 1995; Ergil & Ergil, 2009; Kaptchuk, 2000; Mayor, 2007) have had a great influence on the TCM approach (Figure 1. 10). The TCM view of a human body model is not only restricted to anatomical structures but also focuses its dynamic functions (Barrett, 2011; Matuk, Sc, & Sc, 2006; Ross, 1985).

Figure 1. 10: Yin, Yang and Five Phases

TCM encompasses a combination of energy theories, acupuncture, and herbal prescriptions. Thus, TCM is similar to the European humoral Theory of the Medieval and Renaissance periods (Novella, 2012).

TCM is still considered as a pseudoscience as no scientific evidence have been found to support its basic concepts. As such, the use of TCM is a subject of controversy (Qiu, 2007). An article in 2006 by Prof. Zhang "Farewell to Traditional Chinese Medicine?" argued that TCM should be removed from public healthcare and academia. Stephen Barrett of Quackwatch (2011) wrote that "TCM theory and practice are not based upon the body of knowledge related to health, disease, and health care that has been widely accepted by the scientific community. TCM practitioners disagree among themselves about how to diagnose patients and which treatments should go with which diagnoses. Even if they could agree, the TCM theories are so nebulous that no amount of scientific study will enable TCM to offer rational care" (Barrett, 2011). However, the Chinese Government is still interested in TCM and encourages research into its underlying science, as well as its export and development.

The Modern Concept of Medicine in China

By the 19th Century, Western medicine had developed rapidly and was available to common people so that TCM began to lose its prominence in China. By the 20th century, people realized that Western and TCM were in opposition to each other in their basic philosophies. Although it was believed that TCM could cure diseases, there was logical imperative to prove it. In Western medicinal approaches, scientific proof is paramount. As indicated above, this has proven difficult for TCM.

Nevertheless, TCM does represent an important corpus of "folk-medicine" than could be seen as the product of centuries of "trial-and-error" approaches used by doctors. TCM is therefore attracting considerable interest throughout the world. With the increased export of Chinese

Medicines, it has been easier to apply the modern research ways to detect the molecules, define their mode of action and pharmacokinetic and pharmacodynamics properties. As a result, the Chinese have gained new knowledge of the use of these herbs and also their most effective utilization following clinical and toxicity trials. With increase consumer demand, effective tools have been developed to allow the study of compounds derived from a single plant species. This has led to the establishment of "measures for examining and approving new drugs".

1.4. AIMS AND OBJECTIVES

Natural products have become one of the most important resources for developing new lead compounds and scaffolds. Traditional Chinese medicines are one of the most comprehensive, well-documented traditional and folk medicines in human history. Since the breakthrough in the discovery of Artemisinin from *Artemisia annua*, scientists have conducted comprehensive research into TCM pharmaceutical chemistry and chemical biology. The discovery of artemisinin illustrates how TCM constitutes a great store of knowledge about natural products holds considerable promise. However, the exploitation of natural products represents considerable challenge. These include, for example, adulteration of material due to high demand in the western pharmacology industry, existence of synergistic effects of compounds resulting in activity, and the modes and mechanisms of action are seldom very clear.

The central aim of this thesis is to investigate examples of TCM as sources for new anti-microbials. The specific objectives of this thesis can be summarised as follows:

- Authentication the received TCM samples via DNA barcoding.
- *In vitro* anti-microbial screening of the received TCMs, against a range of clinically relevant pathogens, to ascertain the best candidate TCM for future characterisation.
- Bioassay guided purification and isolation of the natural product from the selected TCM's. and to determine the toxicity of the isolated compounds in *vitro;*
- Establish a rapid metabolomics methodology approach and computational modelling to determine the mode of action for the bioactivity.

General materials and methods

2.1. MATERIAL

Plant materials

The plants and fungi were selected by Prof. Jianying Shen from Institute of Chinese Materia Medica, Beijing, Prof Fuzhong Li from Shanxi Agricultural University, Shaanxi based on interrogation of Chinese TCM databases. The 16 samples were screened based on the ethnopharmacological use confirmed based on internationally available literature (Table I). Information gathered includes Chinese name, common name, traditional benefits, parts and preparation used in TCM.

Microbial strains

Two Gram-negative species (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa,* ATCC 27853), two Gram-positive strains (*Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* USA300 (MRSA)), *Mycobacterium smegmatis* mc^2155, and one fungal strain (*Candida albicans* ATCC 10231) were used for anti-microbial studies.

2.2. METHODS

DNA barcoding

Total genomic DNA of was extracted using a DNeasy Plant Mini kit (Qiagen, UK) in accordance with the manufacturer's instructions. For plants, the *rbcL* sequences where amplified with rbcLa-F: 5′-ATGTCACCACAAACAGAGACTAAAGC-3′ and rbcLa-R: 5′-GTAAAATCAAGTCCACCRCG-3′ primers (CBOL 2009). PCR used a total volume of 20 µL with 10 µL BioMix (BioLine, UK) 1 µl forward primer (10 µM), 1 µL reverse primer (10 µM), and 1 µL of the DNA template (50 ng/µL) and 7 µL HPLC water. PCR involved 1 cycle (94 °C for 3 min), 35 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min), and 1 cycle 72 °C for 7 min.

For fungi, ITS sequences were amplified with ITS8-F: 5'-AGTCGTAACAAGGTTTCCGTAGGTG-3' and ITS4-R 5'-TCCTCCGCTTATTGATATGC- 3' primers. PCR used a total volume of 20 µL with 11.11 µL BioMix (BioLine, UK) 0.89 µl forward primer (10 µM), 0.89 µL reverse primer (10 µM), and 1 µL of the DNA template (50 ng/µL) and 7.11 µL HPLC water. PCR involved 1 cycle (95 °C for 2 min), 35 cycles (95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min), and 1 cycle 72 °C for 10 min (Dentinger, Margaritescu, & Moncalvo, 2010). The resulting 600 bp band was sequenced in both directions on an ABI3730X, using the same primers as used for PCR.

The derived sequence was submitted to BLAST to confirm 100 % identity to *rbcL* and ITS sequences. The *rbcL* and ITS sequences has been submitted to GenBank (Table II).

Crude sample preparation

Dried plant material (aerial parts, roots, leaves, twigs, fruits, rhizome or whole plants) was ground into a powder using a mortar and pestle with liquid nitrogen. Totals of 20 g powder for each TCM were sequentially extracted in 2×500mL of *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) at room temperature with continuous stirring for 24 h, in each solvent. The extracts were filtered, concentrated and stored at 4 ºC until use.

Anti-microbial assay

The plant material was dissolved in 50 % methanol - 50 % water to a final concentration of 5 mg/mL. The antimicrobial activity of all the extracts and isolated compounds was evaluated in 96-well plates by micro-dilution technique. Concentration of the extracts was ranged from 500 µg/mL to 3.125 µg/mL. Serial two-fold dilutions was performed in nutrient broth (*E. coli*, *P. aeruginosa, S. aureus*, MRSA), yeast mould broth (*C. albicans*) and nutrient broth

supplemented with 0.05 % tween 80 and 0.2 % glycerol (*M. smegmatis*). Overnight cultures of the bacterial and *Candida* strains were prepared and ~10^5 cells were incubated in the diluted extracts and compounds. The assays were performed in triplicate and incubated at 37 ^oC for 24 h for *E. coli*, *P. aeruginosa, S. aureus*, MRSA and *C. albicans* and 72 h for *M. smegmatis.* Rifampicin and gentamycin sulphate were used as reference drugs. The absorbance was read in microplates (Hidex Sense) at 630 nm evaluated the bacterial growth. *Mycobacterium* and *Candida* growth was assessed by adding 20 µL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) stain.

Column Chromatography

Column chromatography (CC) was performed using Material Harvest Ltd silica gel 60 (20-230 mesh). Thin layer chromatography (TLC) was performed on Sigma-Aldrich silica gel 60 F_{254} gel plates and visualized under UV light and by spraying sulphuric acid-MeOH (1:1) followed by heating.

Flash column chromatography

Purification of compounds by flash chromatography was performed using a Biotage Flash+ chromatography system with SNAP C18 silica cartridges (30g. 40-63 µm, 60 Å) and fraction collection controlled by photo-diode array (PDA) at 250nm.

Semi-preparative high-performance liquid chromatography (Semi-preparative HPLC)

Chromatographic separation was performed on a reverse phase (RP) ACE 10 C18 250x21.2mm using water with 0.1% formic acid as a mobile phase solvent A and methanol with 0.1% formic acid as a mobile phase solvent B. Each sample was injected using a flow rate of 10 ml/min.

Column oven temperature was set to 40°C. Data was acquired with a photo-diode array (PDA) at 250nm (Dionex).

Ultra-high-performance liquid chromatography-high resolution mass spectrometry (UHPLC-MS)

Fractions were analysed on an Exactive Orbitrap (Thermo Fisher Scientific) mass spectrometer, which was coupled to an Accela Ultra High-Performance Liquid Chromatography (UHPLC) system (Thermo Fisher Scientific). Chromatographic separation was performed on a reverse phase (RP) Hypersil Gold C18 1.9 µm, 2.1 × 150 mm column (Thermo Scientific) using H2O using 0.1 % formic acid (v/v, pH 2.74) as the mobile phase solvent A and ACN: isopropanol (10:90) with 10 mM ammonium acetate as mobile phase solvent B. Each sample (20 µL) was analysed using 0-20 % gradient of B from 0.5 to 1.5 min and then to 100 % in 10.5 min. After 3 min isocratic at 100 % B the column was re-equilibrating with 100 % A for 7 min.

A flow rate of 340 µL/min was used for running the samples. Column oven temperature was set to 60 °C and the data were acquired in both positive and negative ESI, using a heated electrospray ionisation source (HESI). Mass spectra were acquired from 70 to 1400 mass-to-charge ratio (m/z) using a mass resolution of 100,000. The spray voltage was 4 kV for both ionization modes. The temperature of the ion transfer capillary was 370 °C and sheath and auxiliary gas was 30 and 15 arbitrary units, respectively.

The data were recorded using the Xcalibur 2.0.0 software package (Thermo Fisher Scientific). Mass calibration was performed for both ESI polarities before the analysis using a mixture of caffeine, MRFA (L-methionyl-arginyl-phenylalanyl-alanine), Ultramark 1621, sodium dodecyl sulfate (SDS), and sodium taurocholate dissolved in acetonitrile-methanol-water solution with 1 % acetic acid.

Flow injection electrospray high-resolution mass spectrometry (FIE-HRMS):

FIE-HRMS was performed using an Exactive HCD mass analyzer equipped with an Accela UHPLC system (Thermo-Scientific) which generated metabolite fingerprints in both, positive and negative ionization mode, in a single run. Samples (20-µL volume) were injected into a flow of 100-µl.min^{-1} methanol: water (70:30, v/v). Ion intensities were acquired between m/z 50 and 1000 for 3.5 min at a resolution setting of 100,000 (at m/z 200) resulting in 3 (± 1) ppm mass accuracy. ESI source parameters were set according to the manufacturer's recommendations. Raw files were exported to CDF-files, mass aligned and centroided in MATLAB (V8.2.0, The MathWorks) maintaining highest mass accuracy. Mass spectra around the apex of the infusion peak were combined in a single intensity matrix (runs x m/z) for each ion mode. Data from the intensity matrix was log-transformed before further statistical analysis.

Spectroscopy

UV-visible spectra were determined using the Unicam UV-500. NMR spectra were obtained using Bruker Ultra shield-500 NMR spectrophotometer (^{1}H-NMR 500MHz, ^{13}C-NMR 100 MHz) using either $CDCl_3$ or MeOD as the solvent reference.

Antimicrobial screening of Traditional Chinese Medicine

Abstract

In collaboration with Chinese institutions a series of traditional Chinese medicines (TCM) were tentatively linked with antimicrobial activity. This involved searches of the Chinese literature focusing on treatments for various types of infections. Eighteen TCM were targeted (sixteen plant and two fungal TCM) and obtained from commercial sources in China. The identity of the TCM was confirmed through DNA barcoding based on *rbcL* for plant TCM and the Internal Transcribed Sequences (ITS) for fungal TCM followed by sequence comparison with vouchers samples in Genbank. DNA barcoding helped us confirming the species and indicated that some of them that were mislabeled. Extracts of different polarities for selected species were screened for their antimicrobial activity against a range of Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA)) *Mycobacterium smegmatis* bacteria as well as *Candida albicans* using the 96-well plate micro-dilution method. Anti-microbial activity was observed in some TCM extracts against *S. aureus,* MRSA, *M. smegmatis* and *C. albicans.* No anti-microbial effect was observed against gram-negative bacteria. The highest activities were shown by *n*-hexane and dichloromethane extracts of *Dryopteris crassirhizoma Nakai* against *S. aureus* and MRSA (MIC= 6.25 µg/mL and 50 µg/mL respectively) and *n*-hexane fraction of *Oldenlandia diffusa* (Willd.) Roxb. was against *S. aureus* (MIC= 62.5 µg/mL). Our studies indicate the utility of exploring TCM's for drug leads based on records of folk medicine. We also show how rigor is required in sample identification if obtained from commercial sources and confirmation of activities from different sample sources is required before robust conclusions of any intrinsic anti-microbial activities.

3.1. INTRODUCTION

Ethnobotany

With the known side effects of synthetic drugs, there is an increase interest in natural products as they have the advantage of their long-term use by humans. A vast majority of people especially from developing countries rely on traditional medicinal plants for their everyday healthcare. The use of plants by humans followed a trial and error method and the successful results were passed over generations; most often verbally. As a result, ethnobotany plays an important role in recording the traditional medicinal knowledge before it gets lost with the increased "sophistication" of modern societies (Figure 3.1). The successes of ethnobotanical approaches to yield drug leads are well-known. The isolation of anti-malarial drugs quinine from the bark of *Cinchona* species in 1820 by Caventou and Pelletier attracted

scientist to re-evaluate the therapeutically knowledge of all the traditional medicines. Now, most of the 122 plant-derived drugs from 94 plant species currently used in modern science were discovered through ethnobotanical analysis (Gao & Watanabe, 2011; Plengsuriyakarn et al., 2012).

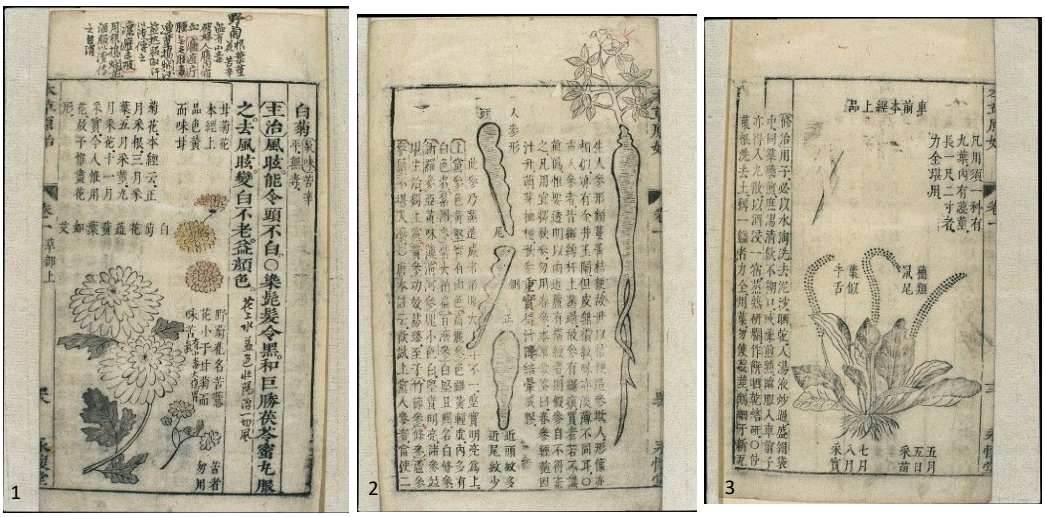


Figure 3.1: Chinese Materia medica.

1: Chrysanthemum; 2: Ginseng; 3: Plantago. Image credit: wellcomeimages.org

Ethnobotanical data can represent a substitute to high throughput screening of bioactives (Rout, Choudary, Kar, Das, & Jain, 2009). Ayurveda (Traditional Indian Medicine) and TCM are considered as two of the most ancient systems in the application of medicinal plants for therapeutic treatments each with histories stretching over several millennia. However, traditional medicines do not represent "off-the-shelf" solutions to current medical need and require validation and quality assurance. Thus, exploitation of traditional medicine requires rigorous scientific assessment of any bioactivities coupled with a consideration of the variation in the bioactives in each sample of the traditional medicine.

Q-Markers in TCM

Chinese medicine quality marker (Q-marker) is a chemical constituent and can be defined, according to the following four basic conditions: (1) quality marker exists in herbs, pieces, extracts, unilateral or compound formulations; (2) quality marker should be analysed through either qualitative and quantitative approach; (3) based on principle of multiple-flavour prescription TCM (such as the King (*Jun*), minister (*Chen*), assistant (*zuo*) and guide (*Shi*), as

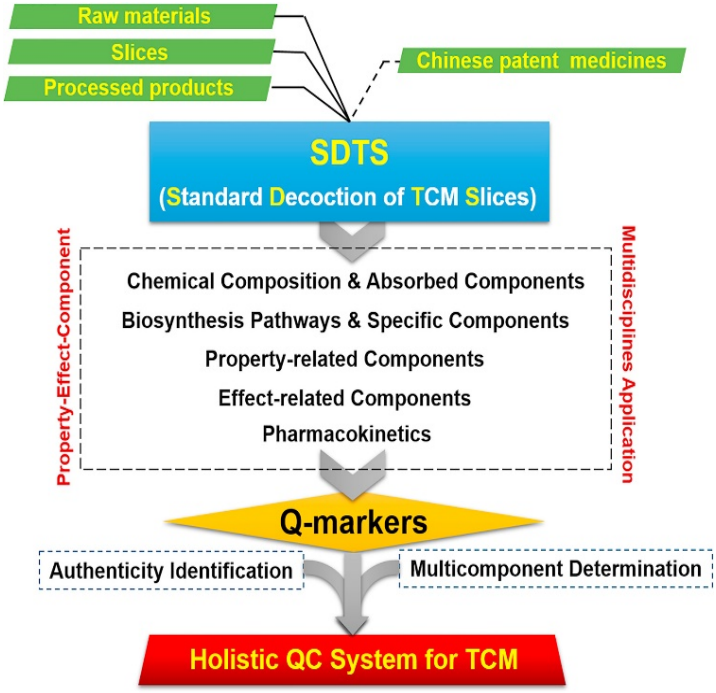


Figure 3.2: A general flowchart for Q-marker-based establishment of holistic quality control system of TCM.

well as the compatibility of TCM and modern pharmacological study, the drug effect (such as effectiveness and safety) should be demonstrated to be associated with the identified quality marker, (4) the quality marker is a chemical substance that are transferable and traceable in the process of production and preparation (Figure 3.2).

The relevance of Q-markers for TCM quality control is exemplified by ginseng. Ginseng is ranked among the most popular natural products worldwide (Qi, Wang, & Yuan, 2011). Three Panax species, *P. ginseng* (Asian ginseng), *P. quinquefolius* (American ginseng), and *P. notoginseng* (Sanchi ginseng), are largely consumed as the healthcare products, dietary supplements, and herbal medicines (Yang, Hu, Wu, Ye, & Guo, 2014). Establishment of the Q-markers that ensure the authenticity of Ginseng Radix et Rhizoma (Panax ginseng; Ren-Shen) is difficult, since the chemical difference among the congeneric species (such as *Panax quinquefolius* and *Panax notoginseng*) and different parts (the root/rhizome, stem/leaf, flower, berry, and seed) needs to be clarified. Given the bioactivity and specificity ginsenosides, saponins and flavonoid (Park, Rhee, & Lee, 2005; Yang et al., 2014), were mainly used to establish the quality markers or the exact identification among the root/rhizome, stem/leaf, flower, and berry of *P. ginseng*.

Challenges with the quality standards

Many factors, such as the species, cultivation/production area conditions (geo-herbalism), harvesting, processing, transportation/storage conditions, extraction/purification, ADME (absorption, distribution, metabolism, and excretion), and interaction of diverse components (Liu et al., 2016), can affect the quality of TCM. The chemical composition and content of markers in TCM medicinal materials, extractives, products, and formulae, are measured by Ultraviolet-visible spectroscopy (UV), thin layer chromatography (TLC), High Performance Liquid Chromatography (HPLC) (or Ultra-High-Performance Liquid Chromatography

(UHPLC)), Gas chromatography (GC), Liquid chromatography-mass spectrometry (LC-MS), or Gas chromatography-mass spectrometry (GC-MS), for authenticity identification and quality evaluation. The difficulties of quality assessment of TCM products can be listed as following: (1) it is associated with Good Agricultural Practice, Good Collection Practice (GACP), and Good Supplement Practice (GSP); (2) specific eco- geographic regions (EGRs) of botanical raw materials is of particular importance; (3) how to reduce the variability from the plant raw materials to production process; and (4) based on well-known mechanism of action (MOA) and TCM theory, potency evaluation of bioactive response become flexible for control of the manufacturing process. Based on the above, it is suggested that: (i) TCM products are complex with multiple chemical components (known and unknown active components) and natural variations; (ii) generally TCM product is the mixture of the active pharmaceutical ingredients (API) and inactive ones from raw materials of herbs; (iii) TCM products are very difficult to satisfy the identical requirement of quality, safety and efficacy as that of chemical drugs; and (iv) the principle of multiple-flavour prescription in TCM is also far different from the strategy used in Western medicine. The intrinsic "multicomponent and multi-target" feature of TCM necessitates the establishment of a unique quality and bioactivity evaluation system, which is different from that of the Western medicine (Yang et al., 2017).

This chapter will aim to use the expertise of our Chinese collaborators to identify potentially anti-microbial TCM and establish an analysis pipeline (Figure 3.3) which would include the confirmation of their identities to screening for bioactivities.

3.2. RESULTS

In order to screen followed by isolation of bioactive from of the selected TCMs, we have instigated a pipeline where interrogation of Chinese sources can indicate usage of particular TCM in diseases where anti-microbial activities could be prominent can indeed, yield the hypothesised (Figure 3.3). It involves the following steps:

- Initial screening of the crude extract
- Sequence of bioassay guided purification
- Structure elucidation of isolated compounds by spectroscopic techniques namely High-Performance Liquid Chromatography (HPLC) and 2D Nuclear Magnetic Resonance (NMR)
- Assessment of biological activities of purified compounds *in vitro*
- Mode of action studies using different omics technologies
- Preclinical studies to understand pharmacokinetics and mechanism of action of the selected bioactive compound

The 16 different genera used in TCM were selected based on literature research. There common name, traditional ways of consumption, physiological portions used as medicine and already studied biological activities are listed in

Table **I** and Table II

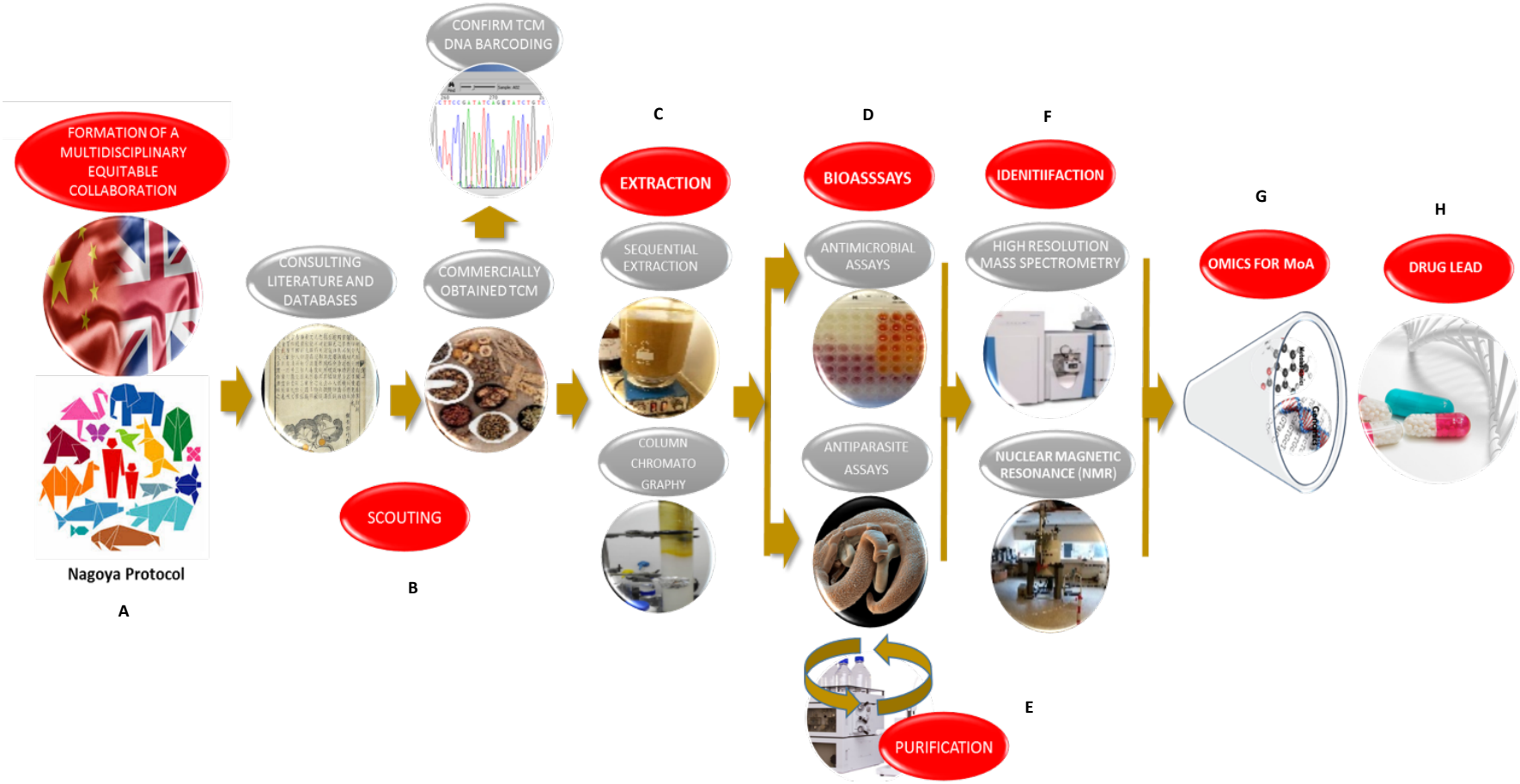


Figure 3.3: Pipeline for TCM bioactive discoveries.

(A): Collaborators with expertise in TCM are identified and an agreement in line with the Nagoya protocols are established. **(B) SCOUTING:** The folk medicinal literature is consulted to identify TCM which may have relevant bioactivities; (e.g. countering infections as a source of anti-microbials). **(C) EXTRACTION**: The TCM are sourced and chemicals are extracted using various solvents. **(D) BIOASSAYS:** The extracted are screened for activities against clinically relevant bacterial based on establishing the minimum inhibitory concentration (MIC) using a 96 -well plate dilution methods. Anti-parasite activities are based on screens against *Schistosoma mansoni*. **(E) PURIFICATION:** involved sequential bioactivity guided fractionation to eventually yield a pure product. **(F) IDENTIFICATION:** was based on high resolution mass spectroscopy and nuclear magnetic resonance (NMR). **(G):** Further derivatization of the product is based on **'OMIC DETERMINATION OF MODE OF ACTION (MoA).** The ultimate aim of the pipeline is to define a possible **DRUG LEAD(H).**

Table I: Ethnobotanical data of the plants studied.

N.A: no literature on bioactivity found.

Plants	Chinese name	Common name	Part used in TCM	TCM Uses	TCM consumption	Scientifically proved properties	References
***Artemisia annua* L.**	*qinghao*	Sweet wormwood	Aerial parts	Fever	Decoction, squeezed juice	Anti-malarial Anti-inflammatory Anti-tumour Allelopathic	(Ho, Peh, Chan, & Wong, 2014a; J.-B. Jiang, Guo, Li, Kong, & Arnold, 1982; Kuhn & Wang, 2008)
***Polygala tenuifolia* Poir.**	*Yuan zhi*	Seneca snakeroot,	Root	Expels Phlegm Reduces abscesses and swellings	Decoction	Hypoglycemic activity Anti-influenza Promotes neurogenesis Anti-inflammatory Anthelmintic	(H.-J. Kim, 2019; C. Lu, Zhang, Ji, & Wang, 2012; She et al., 2011)
***Fritillaria cirrhosa* D.Don**	Chuan bei Mu	Yellow himalayan fritillary	Bulbs	Expels Phlegm Reduces swellings Arrest cough Pulmonary tuberculosis	Decoction	Anti-inflammatory Anti-tumour Antitussive Antibacterial	(Wang et al., 2014; Wang et al., 2014; Wang, Yang, Du, Li, & Wang, 2016; Wang et al., 2011)
***Fritillaria thunbergii* Miq.**	Zhe bei mu	Fritillaria Bulb	Bulbs	Expels Phlegm Reduces swellings Arrest cough Pulmonary tuberculosis	Decoction	Anti-*H. pylori* activity Anti-thrombin Antioxidant	(Li, Xu, Zhang, Liu, & Tan, 2005; Shin, Yong-Kyu; Jang, Han-Su; Kim, Jee-In; Sohn, 2009)

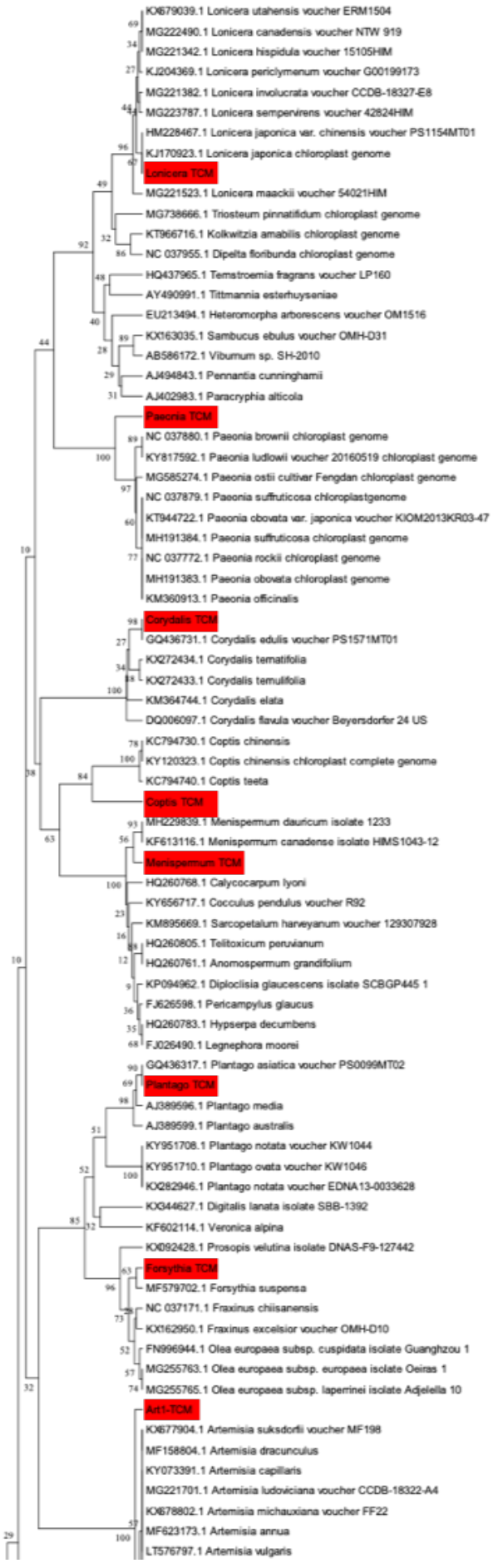
***Pseudostellaria heterophylla* (Miq.) Pax**	Hai er shen	False starwort	Root	Tonify Nourish Pneumonia Lung diseases	Decoction	Antitumor	(Wang & Ng, 2006; Wong, Leung, Fung, Fung, & Choy, 1994)
***Paeonia lactiflora* Pall.**	Bai shao	White peony root	Root	Irregular menstruation Abdominal pain Liver diseases	Herbal teas or encapsulated as a supplement	Anti-inflammatory Analgesic Anti-*H. pylori* activity	(He & Dai, 2011; Ngan, Moon, Kim, Shibamoto, & Ahn, 2012; Ngan, Moon, Shibamoto, & Ahn, 2012; Wang, Chen, & Xu, 1988)
***Lonicera japonica* Thunb.**	Rěn dōng téng or īn yín huā	Golden-and-silver honeysuckle	Flower	Reduce body heat Reduces abscesses Reduces Sore throat Dysentery Polydipsia	Decoction on,tea,pills	Antibacterial Antiviral Cytoprotective Anti-inflammatory Antioxidant Hepatoprotective	(Rumalla, Avula, Zhao, Smillie, & Khan, 2011)
***Forsythia suspense* (Thunb.) Vahl**	Pinyin, liánqiào	Weeping forsythia or golden-bell)	Fruit	Reduce body heat Reduces abscesses Reduces Sore throat Sucutaneous nodule	Decoction	Hepatoprotective Anti-inflammatory Antioxidant Anticancer Antiviral Antibacterial	(Guo, Lin, & Wang, 2015; Kuo, Chen, Yang, Lin, & Peng, 2014; Ozaki, Rui, & Tang, 2000; H. Qu, Zhang, Wang, Li, & Sun, 2008; X. yan Qu et al., 2016; Wen, Huifu, & Hao, 2010; Zhang, Song, Ren, & Xu, 2002; Zhang, Miao, Yan, Sheng, & Ji, 2018)
Oldenlandia diffusa or Herba Hedyotidis Diffusae	Bai Hua She She Cao	White flower snake-tongue grass	Whole plant	Detoxifying Sore throat Urinary infection Jaundice Snakebite Sore Swellings	Decoction	Bronchitis Sore throat Urethral infection Anticancer Anti-inflammatory	(Lin et al., 2010, 2011; Niu & Meng, 2013; Ye, Liu, Zhang, & He, 2015)

***Astragalus membranaceus* Fisch. ex Bunge**	Huang Qi	Milk vetch root	Root	Tonify Edema Sores Ulcers Anemia	Decoction, Bake	Hepatoprotective Anti-inflammatory Antiviral Antioxidant Antidiabetic	(Agyemang et al., 2013; Jin, Zhao, Huang, & Shang, 2014)
***Coptis chinensis* Franch.**	Duǎn è huánglián	Goldthread	Rhizome	Reduce body heat Vomiting Abscesses Boils Burns	Decoction, powder, pills, Fry, External application	Antihypertensive Antibacterial Antioxidative Anti-inflammatory	(Jung et al., 2009; J. M. Kim, Jung, Choi, & Lee, 2010; Kong et al., 2009; Tsai, Chen, & Lo, 2008)
***Corydalis edulis* Maxim.**	Yan Hu Suo	Common Rhizoma Corydalis	Rhizome	Activate and promote blood flow	Powder	Antidiabetic	(Zheng et al., 2017)
***Plantago asiatica* L.**	Che Qian Zi	Plantago Seeds, Asian Plantain Seeds	Seed	Promotes urination Improves vision Clear phlegm Diarrhea	Decoction, Food	Anti-inflammatory Angiotensin-converting enzyme inhibition	(Geng, Yang, Chou, & Wang, 2010; Q. Zhou et al., 2013)
***Menispermum dauricum* DC.**	Bian Fu Ge Gen	Asiatic Moonseed Root	Root	Sore throat Jaundice Lung-heat cough Dysentery Detoxify Rheumatism	Decoction	Anti-tumour Cardiovascular effects Anti-arrhythmic	(M. Lin et al., 2013; Qian, 2002)
***Chrysanthemum lucidum* Nakai**	Ye ju hua	Chrysanthemum	Flower	Ulcers Abscess Red eyes Cold and flu Headache and dizziness	Decoction	N.A	N.A

***Dryopteris crassirhizoma* Nakai**	Guan Zhong	Wood Fern, Dryopteris Root	Rhizome	Verminosis Flu	Raw, Stir-fried	Antibacterial Anti-inflammatory Cytotoxic Antiviral Vermifuge Anti-tumour	(Kapadia et al., 1996; Kwon et al., 2007;Lee, Kim, & Lee, 2009; Lee, Miyashiro, Nakamura, & Hattori, 2008; Magalhães et al., 2010; Yang et al., 2013; Zhao et al., 2014)

Table II: Ethnobotanical data of the fungi studied.

Fungi	Chinese name	Common name	Part used in TCM	TCM Uses	TCM consumption	Scientifically proved properties	References
Polyporus umbellatus	Zhu ling	Grifola umbellata	Whole	Dysuria Edema Diarrhea	Decoction	Diuretic Anticancer Hepatoprotective Immuno-enhancing Antimicrobial	(Xingqun Li & Xu, 2011; Xinqun Li, Xu, & Chen, 2010; Sun & Zhou, 2014; G. Zhang, Zeng, Han, Wei, & Huang, 2010; G. Zhang et al., 2011)
Ganoderma lucidum	Lingzhi	Ganoderma, lucid ganoderma, reishi mushroom	Whole	Tonify Nourish Insomnia Amnesia Relieve cough Dyspnea	Decoction, powder, Pills, Alcoholic preparation	Antitumor Hepatoprotective Antidiabetic Antibacterial Antiviral	(Gao, Lan, Dai, Ye, & Zhou, 2004; Gao et al., 2002; Suay et al., 2000; Wang, Xi, Li, Wang, & Yao, 2012; Wasser, 2005)


KX679039.1 Lonicera utahensis voucher ERM1504
MG222490.1 Lonicera canadensis voucher NTW 919
MG221342.1 Lonicera hispidula voucher 15105HIM
KJ204369.1 Lonicera periclymenum voucher G00199173
MG221382.1 Lonicera involucrata voucher CCDB-18327-E8
MG223787.1 Lonicera sempervirens voucher 42824HIM
HM228467.1 Lonicera japonica var. chinensis voucher PS1154MT01
KJ170923.1 Lonicera japonica chloroplast genome
Lonicera TCM
MG221523.1 Lonicera maackii voucher 54021HIM
MG738666.1 Triosteum pinnatifidum chloroplast genome
KT966716.1 Kolkwitzia amabilis chloroplast genome
NC 037955.1 Dipelta floribunda chloroplast genome
HQ437965.1 Ternstroemia fragrans voucher LP160
AY490991.1 Tittmannia esterhuyseniae
EU213494.1 Heteromorpha arborescens voucher OM1516
KX163035.1 Sambucus ebulus voucher OMH-031
AB586172.1 Viburnum sp. SH-2010
AJ494843.1 Pennantia cunninghamii
AJ402983.1 Paracryphia alticola
Paeonia TCM
NC 037880.1 Paeonia brownii chloroplast genome
KY817592.1 Paeonia ludlowii voucher 20160519 chloroplast genome
MG585274.1 Paeonia ostii cultivar Fengdan chloroplast genome
NC 037879.1 Paeonia suffruticosa chloroplastgenome
KT944722.1 Paeonia obovata var. japonica voucher KIOM2013KR03-47
MH191384.1 Paeonia suffruticosa chloroplast genome
NC 037772.1 Paeonia rockii chloroplast genome
MH191383.1 Paeonia obovata chloroplast genome
KM360913.1 Paeonia officinalis
Corydalis TCM
GQ436731.1 Corydalis edulis voucher PS1571MT01
KX272434.1 Corydalis ternatifolia
KX272433.1 Corydalis temulifolia
KM364744.1 Corydalis elata
DQ006097.1 Corydalis flavula voucher Beyersdorfer 24 US
KC794730.1 Coptis chinensis
KY120323.1 Coptis chinensis chloroplast complete genome
KC794740.1 Coptis teeta
Coptis TCM
MH229839.1 Menispermum dauricum isolate 1233
KF613116.1 Menispermum canadense isolate HIMS1043-12
Menispermum TCM
HQ260768.1 Calycocarpum lyoni
KY656717.1 Cocculus pendulus voucher R92
KM895669.1 Sarcopetalum harveyanum voucher 129307928
HQ260805.1 Telitoxicum peruvianum
HQ260761.1 Anomospermum grandifolium
KP094962.1 Diploclisia glaucescens isolate SCBGP445 1
FJ626598.1 Pericampylus glaucus
HQ260783.1 Hypserpa decumbens
FJ026490.1 Legnephora moorei
GQ436317.1 Plantago asiatica voucher PS0099MT02
Plantago TCM
AJ389596.1 Plantago media
AJ389599.1 Plantago australis
KY951708.1 Plantago notata voucher KW1044
KY951710.1 Plantago ovata voucher KW1046
KX282946.1 Plantago notata voucher EDNA13-0033628
KX344627.1 Digitalis lanata isolate SBB-1392
KF602114.1 Veronica alpina
KX092428.1 Prosopis velutina isolate DNAS-F9-127442
Forsythia TCM
MF579702.1 Forsythia suspensa
NC 037171.1 Fraxinus chiisanensis
KX162950.1 Fraxinus excelsior voucher OMH-010
FN996944.1 Olea europaea subsp. cuspidata isolate Guanghzou 1
MG255763.1 Olea europaea subsp. europaea isolate Oeiras 1
MG255765.1 Olea europaea subsp. laperrinei isolate Adjelella 10
Art1-TCM
KX677904.1 Artemisia suksdorfii voucher MF198
MF158804.1 Artemisia dracunculus
KY073391.1 Artemisia capillaris
MG221701.1 Artemisia ludoviciana voucher CCDB-18322-A4
KX678802.1 Artemisia michauxiana voucher FF22
MF623173.1 Artemisia annua
LT576797.1 Artemisia vulgaris

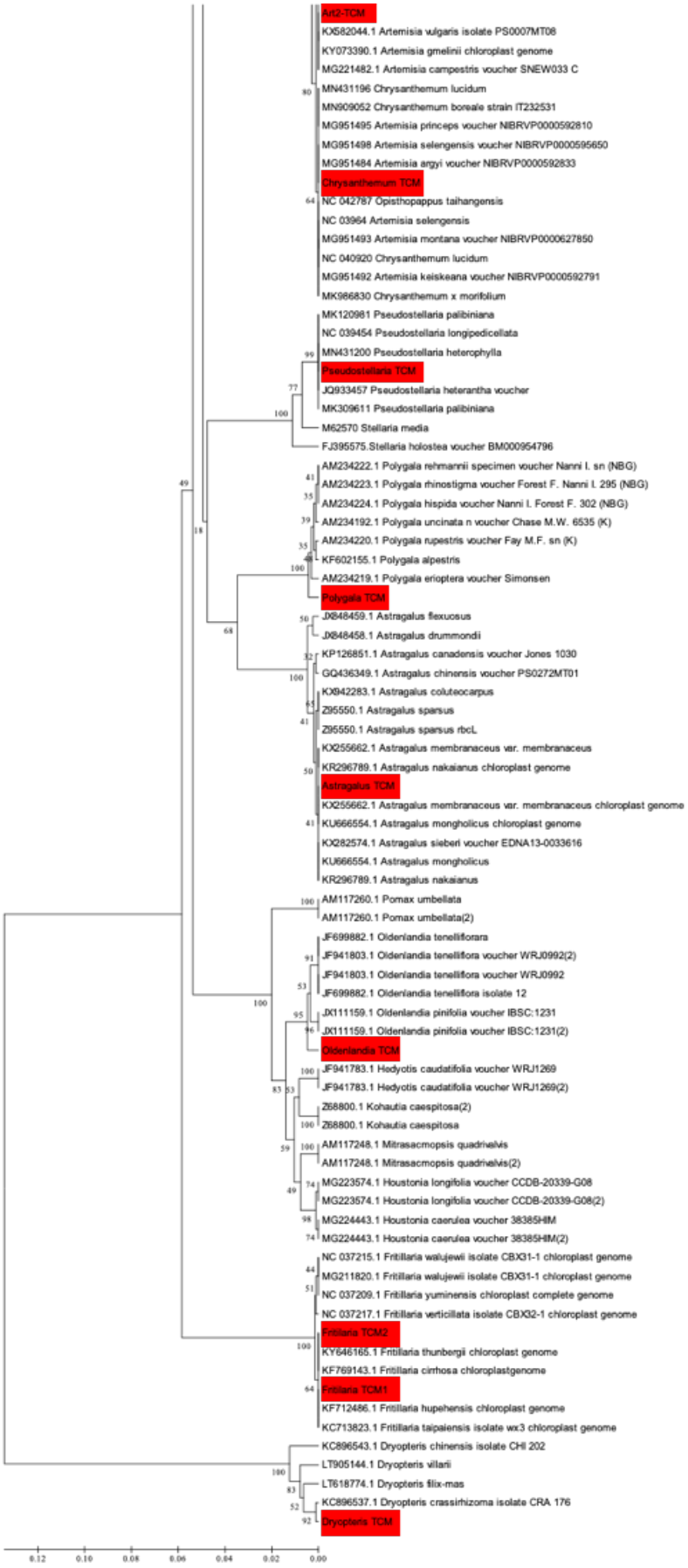


Figure 3.4: Phylogenetic relations between of the selected TCM plants and associated vouchers based on *rbcL* sequence variation

As the plants and fungi were collected from commercial sources, it was essential to authenticate the sample to avoid adulteration. DNA barcoding was used in this study to authenticate the plant species. The plant DNA barcode regions *rbcL* (ribulose-bisphosphate carboxylase) and fungal barcode region the internal transcribed spacer (ITS) were used. The barcode sequences were further submitted online to GenBank database using the Bankit tool (Figure 3.4 and Figure 3.5). Their accession numbers are shown in Table III.

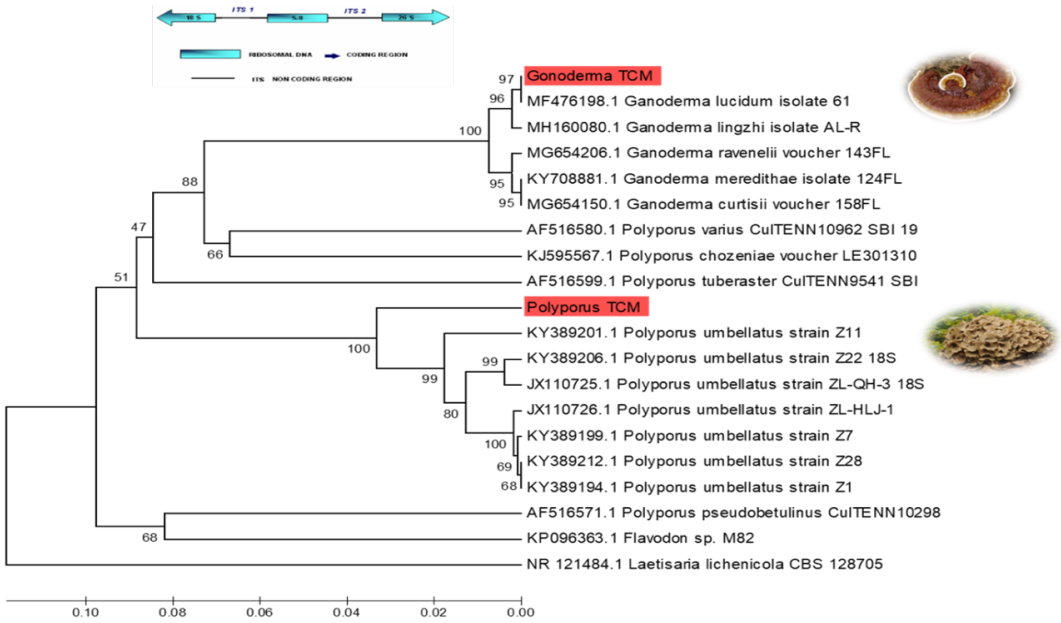


Figure 3.5: Phylogenetic relation of the selected fungi TCM and associated vouchers based on ITS sequence variation

The extracts were prepared by sequentially extracting with solvents based on polarity resulted in 64 extracts. The MIC values of the extracts in context to the respective bacteria are summarized in Table III. The antimicrobial activity assessments were undertaken in triplicate and no variation in results was observed. The type of extracts in rank of their activity are *n*-hexane, DCM, EtOAc and MeOH. Extracts showed activity against *S. aureus*, MRSA, *M. smegmatis* and *C. albicans*, while were not active against *E. coli* and *P. aeruginosa.* The *n*-hexane and ethyl acetate extract of *Dryopteris crassirhizoma* and *n*-hexane extract of *Oldenlandia diffusa* were particularly effective against *S. aureus* and MRSA with very low

MICs. Extracts showed no activity against Gram-negative bacteria even if displaying some activity against Gram-positive, fungi and *M. smegmatis.* The poor results obtained for Gram-negative bacteria could reflect the hydrophobic outer membrane creating a permeability barrier for different antibiotics mainly hydrophilic compounds (Poole, 2002) (Nikaido, 1994).

Table III: Antimicrobial screening of authenticated TCMs (MIC µg mL^{-1})

Plant species	Accession numbers	Part	Solvents	*E. coli*	*P. aeruginosa*	*S. aureus*	MRSA	*M. smegmatis*	*C. albicans*
Artemisia annua	MH051919	*Aerial parts*	*n*-Hexane	>500	>500	500	500	500	500
			DCM	>500	>500	>500	>500	>500	500
			EtOAc	>500	>500	>500	>500	>500	500
			MeOH	>500	>500	>500	>500	>500	500
Polygala tenuifolia	MN431187	*Root*	*n*-Hexane	>500	>500	>500	>500	>500	>500
			DCM	>500	>500	>500	>500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Fritillaria cirrhosa	MT354770	*Bulbs*	*n*-Hexane	>500	>500	>500	>500	250	>500
			DCM	>500	>500	>500	>500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Fritillaria thunbergii	MN431190	*Bulbs*	*n*-Hexane	>500	>500	>500	>500	500	>500
			DCM	>500	>500	>500	>500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
	MN431200	*Roots*	*n*-Hexane	>500	>500	>500	>500	>500	>500

Pseudostellaria heterophylla			DCM	>500	>500	>500	>500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Paeonia lactiflora	MN431191	*Roots*	*n*-Hexane	>500	>500	>500	>500	>500	>500
			DCM	>500	>500	>500	>500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	500
Ganoderma lucidum	MN431189	*Whole*	*n*-Hexane	>500	>500	>500	>500	>500	>500
			DCM	>500	>500	250	>500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Lonicera japonica	MN431192	*Dried flowers*	*n*-Hexane	>500	>500	500	>500	500	>500
			DCM	>500	>500	500	>500	>500	>500
			EtOAc	>500	>500	250	250	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Forsythia suspensa	*No accession number obtained*	*Fruit shell*	*n*-Hexane	>500	>500	>500	>500	>500	>500
			DCM	>500	>500	500	500	>500	>500
			EtOAc	>500	>500	500	500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500

Oldenlandia diffusa or Herba Hedyotidis Diffusae	MN431193	*Whole plant*	*n*-Hexane	>500	>500	62.5	125	500	>500
			DCM	>500	>500	>500	500	>500	>500
			EtOAc	>500	>500	250	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Astragalus membranaceus (Fisch.) Bunge (AM)	MN309697	*Root*	*n*-Hexane	>500	>500	>500	250	500	>500
			DCM	>500	>500	>500	500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	500	>500
Coptis chinensis	MN431186	*Rhizome*	*n*-Hexane	>500	>500	>500	>500	250	>500
			DCM	>500	>500	250	500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	125	500	>500	250
***Corydalis edulis* Maxim.**	MN431194	*Rhizome*	*n*-Hexane	>500	>500	>500	>500	>500	>500
			DCM	>500	>500	500	500	>500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Plantago asiatica	MN431195	*Seed*	*n*-Hexane	>500	>500	>500	>500	500	>500
			DCM	>500	>500	500	>500	500	>500
			EtOAc	>500	>500	>500	>500	>500	>500

			MeOH	>500	>500	>500	>500	>500	>500
Menispermum dauricum	MN431199	*Root*	*n*-Hexane	>500	>500	250	250	250	>500
			DCM	>500	>500	500	250	250	>500
			EtOAc	>500	>500	500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Chrysanthemum lucidum	MN431196	*flower*	*n*-Hexane	>500	>500	>500	>500	500	>500
			DCM	>500	>500	500	>500	500	>500
			EtOAc	>500	>500	500	>500	500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Dryopteris crassirhizoma	MN431197	*Rhizome*	*n*-Hexane	>500	>500	3.125	3.125	500	>500
			DCM	>500	>500	25	50	500	>500
			EtOAc	>500	>500	>500	>500	>500	>500
			MeOH	>500	>500	>500	>500	>500	>500
Rifampicin		-	-			-	-	1.14	-
Gentamycin Sulphate		-	-	1.6	3.13	3.13	3.13	-	-

3.3. DISCUSSION

TCM are available to be used by consumers in a variety of forms such as capsules, extracts, teas, tinctures and traditional formulations. Several agencies have provided guidance prior to its use for research purposes. Specific journals and their editors have provided specifications on the characteristics of the targeted natural products before publication is permitted (Kellogg et al. 2019). These are that: (1) the identities of major metabolites, especially those that may be biologically active needs to be defined; (2) the *in vitro* or *in vivo* targets and activities require accurate definition; (3) the results need to fill a current knowledge gaps; and that (4) prior consumer usage of the TCM was study (Kellogg et al. 2019). It is suggested that the product selected must collected from the same commercial products used by the target population. Products can be either collected from (1) the regional or national herbaria , who have historical records of the specimen, (2) research groups who can prepare or grow the respective specimen, (3) from a commercial supplier or (4) collected from the wild condition.

Once samples are collected, the first and the most important step is their authentication. Samples can be authenticated by multiple means, including examination of the morphological and microscopic characteristics, DNA barcoding, chemotaxonomy, chemical fingerprints and untargeted metabolomics (Kellogg et al. 2019). In our experiments, we authenticated the commercially obtained samples using DNA barcoding by sequencing specified PCR fragments. The plant DNA barcode regions *rbcL* is the most characterized plastid coding sequence in GeneBank with wide representation from all major groups. In polymerase chain reaction (PCR), *rbcL* gene had shown higher amplification efficiency and sequencing success rate compared to other selected plant characterization genes (Parveen, Singh, Raghuvanshi, Pradhan, & Babbar, 2012; Pawlowski et al., 2012). Due to this unique property of *rbcL* gene, it is ideal for studying the genetic

variations of the plants species (Hamdan, Abd Samad, Hidayat, & Mohd Salleh, 2013). White *et al.*, 1990 suggested that fungal ITS are remarkably robust, works with the vast majority of fungi The ITS varies in length considerably among major taxonomic groups and gives superior

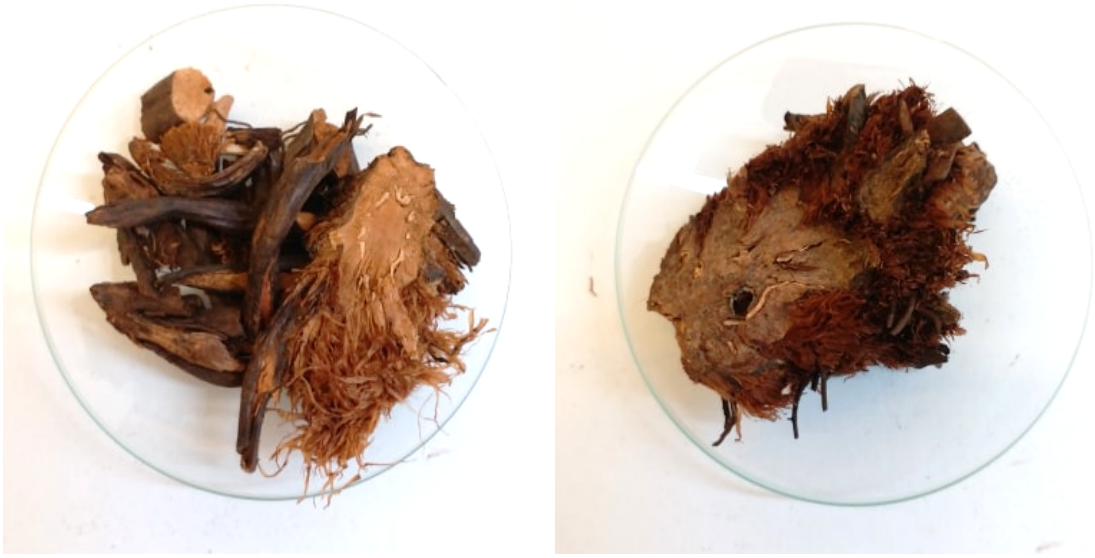


Figure 3.7: Two sets of *Dryopteris crassirhizoma* supplied to us from different provinces of China.

DNA barcoding confirmed as (A) *Dryopteris crassirhizoma*, (B) *Plantago lanceolata*

resolution in those groups with longer amplicons. Fungal ITS is indel rich, which makes it useful for the development of molecular diagnostics involving taxon specific oligonucleotides(Seifert, 2009). Here conserved DNA sequences (used as primer binding site) straddle a hypervariable region where variation may be associated with specific genera or species. DNA barcoding is an effective tool for fresh, dried, or powdered material, where intact DNA is still present. However, it fails in case of a processed products where either the DNA is absent or degraded in the process. However, in our hands the value of a DNA barcoding validation step was demonstrated. For example, we were provided one of the plant sample stating it to be *Forsythia suspense,* but once the sample was barcoded the sequence resembled *Syringa yunnanensis* (Figure 3.8: Received material tagged as *Forsythia suspensa*); for which no TCM properties have been described. Samples labelled *Polygala tenuifolia* resembled

Polygala rehmannii. Additionally, when we received a second set of *Dryopteris crassirhizoma* this was barcoded to be *Plantago lanceolate* (Figure 3.7).

Figure 3.8: Received material tagged as *Forsythia suspensa*

Once the samples are authenticated, the next step in our analysis pipeline (Figure 3.3) was to prepare extracts from the samples. In our experiment, samples were extracted using gradient of solvent resulting in a range of chemical products with difference in polarity. The solvents were later evaporated from the extracted products for long-term storage of the samples.

These extracts were then screened for their antimicrobial potential. The susceptibility of pathogen against an antibiotic can be determined using MIC. The MIC represents the lowest concentration of antimicrobials needed to inhibit the bacterial growth. Considering different criteria and the reference drugs, extracts with ≤250µg/mL were considered as active for this study. We selected a panel of hospital derived bacterial species including Gram-positive, Gram-negative bacteria, fungi and multi-drug-resistant strains. Several extracts showed no activity against Gram-negative bacteria than those found for Gram-positive, fungi and *M. smegmatis*. The poor results obtained for Gram-negative bacteria can be justified by their

hydrophobic outer membrane that creates a permeability barrier for different antibiotics mainly hydrophilic compounds (Baptista, Bhowmick, et al. 2018).

The bioactivity screens suggested further variation in the levels of active compounds. Thus, we were donated *A. annua* from two different Chinese groups (Figure 3.9). DNA barcoding confirmed that both are Artemisia species, but on testing antimicrobial activity, we discovered that plant 2 in Figure 3.4, was completely inactive. We concluded that the difference in the activity could be because of the constituents of the material. As from the Figure 3.9, we can see that plant 1 contains more of leaves and aerial part of the plant while plant 2 have more of twigs and old leaves. There is a long history of use of the aerial parts and leaves of *A. annua* for medicinal purposes in certain Asian countries (Fallis, 2013) but our data suggested this could have a lower content of anti-mycobacterial compounds.

Other positive anti-microbial activities were also observed. *Fritillaria chirriosa* is known to hydrate lungs and treat phlegm. *Fritillaria* bulbs are frequently used in a blend formulation for acute bronchitis and tuberculosis, such as San She Dan Chuan Bei Mu Ye and Chuan Bei Pi

Figure 3.9: Two different types of *Artemisia annua*.

1: From China Academy of Chinese Medical Sciences, Beijing, 2: From local TCM shops from China

Pa Lu. Beyond such traditional uses pharmacological studies stated anti-inflammatory,

antitumor, antitussive and antibacterial activity (D. D. Wang et al. 2014). Our results confirmed such activities and showed an MIC of 250 µg/mL of the n-hexane extract against *M. smegmatis,* suggesting that our approach was appropriate.

Oldenlandia diffusa is used in a TCM to treat hepatitis, tonsillitis, sore throat, appendicitis, urethral infection and malignant tumours of the liver, lung and stomach. The herbs have been reputed to have antitumor, immunomodulatory, anti-mutagenic, anti-inflammatory, hepatoprotective, anti-oxidative and neuroprotective properties (Liang et al. 2008). Our studies provided a new description of antimicrobial activity in the herb (Table III).

Coptis chinensis a used the treatment of diarrhea, fever and eczema. Phytochemical studies indicate antibacterial, antiviral, anti-inflammatory and antitumor activities. In this case, activity seem to be based on berberine which has a reported MIC in the range of 32 to 128 µg/mL against MRSA (Zuo et al. 2012). Our results show a MIC of 250 µg/mL of the n-hexane extract showed against *M. smegmatis*, DCM extract agains*t S. aureus* and methanol extract against *C. albicans* respectively and a MIC of 125 µg/mL against MRSA.

Menispermum dauricum rhizome are well known traditional medicine in China used to treat sore throat, jaundice, lung-heat cough, dysentery, detoxify and rheumatism. Phytochemical studies have reported its anticancer properties (Zhou et al. 2019) and antimicrobial properties (Kang et al. 2013). Our results show a MIC of 250 µg/mL of n-hexane extract against *M. smegmatis, S. aureus,* MRSA and the DCM extract against MRSA and *M. smegmatis.*

The literature has reported antimicrobial activity of different extracts of *Ganoderma lucidum.* This species is one of the most famous traditional medicinal mushrooms, being used as functional food and in traditional medicines (Heleno et al. 2013). Our work indicated activities similar to those previously reported with an MIC of 250 µg/mL against *S. aureus,* which was considered to be quite poor activity.

Dryopteris crassirhizoma is commonly used as an anti-viral in TCM. It also has anticancer, antioxidant, antibacterial, anti-inflammatory, antimalarial and antitumor activities (Na et al. 2003). Our data shows that the n-hexane extract active against *S. aureus* (MIC of 6.25 µg/mL) and against MRSA (MIC of 6.25 µg/mL), DCM extract again*st S. aureus* (MIC of 25 µg/mL) and against MRSA (MIC of 50 µg/mL). The plant has been previously reported to active against different MRSA isolates (Kwon et al. 2007).

3.4. CONCLUSION

The quality, precise identification and reliability in the plant species from where the natural product is obtained is very critical step for successful innovative drug discovery. The complex chemical composition of plants or TCM material and variation in source can lead to batch-to-batch inconsistency. Genomic techniques such as DNA barcoding are established techniques that rely on sequence diversity in short, standard *rbcL* regions (400–800 bp) and internal transcribed spacer for species-level identification.(Ganie, Upadhyay, Das, & Prasad Sharma, 2015) Thus, we have attempted to incorporate some quality assessments based on DNA barcoding and assessments of more than one batch as variation could be importance as bioactivities are likely not be uniformly present in every batch.

Based on these studies, it is possible to suggest that TCM could yield new medicines against infectious diseases. The authentication and promising activity of some of the plant extracts will be taken forward in subsequent chapters and the active compounds characterised.

4

Exploring the anti-microbial potential of *Artemisia annua*

Abstract

The development of anti-parasitic artemisinin from *Artemisia annua* L. is an example of how traditional Chinese medicine (TCM) may be exploited to meet a recognized need. We systemically investigated *A. annua* for its antimicrobial activity, which has not previously reported. In this study, we assessed *A. annua* as a source of bioactive natural products for anti-mycobacterial activity.

Anti-mycobacterial activity-guided purification of the *A. annua* leaf on a silica gel column and structure elucidation using UHPLC-HRMS and NMR resulted in the identification of active compounds. Crude extracts, isolated compounds and artemisinin were assessed against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycolicibacterium smegmatis* strains by serial micro dilution method (31.25-1000 µg/mL). Anthelmintic activity of isolated compounds and artemisinin against *Schistosoma mansoni* was analyzed by the Roboworm anthelmintic discovery platform. The isolated compounds were tested for synergistic effects against *Mycobacterium*. We also used a molecular docking approach to investigate the interactions between selected anti-mycobacterial compounds and proteins involved in vital physiological functions in *M. tuberculosis*, namely MtPks13, MtPknB, MtPanK, MtKasA, MtInhA and MtDprE1.

Identity of *A. annua* was confirmed by *rbcL* DNA barcoding, bioactive compounds were purified and identified as deoxyartemisinin and artemisinic acid. Artemisinic acid (MIC 250 µg/mL) was more effective in comparison to deoxyartemisinin (MIC 500 µg/mL) and artemisinin (MIC 1000 µg/mL) against *M. smegmatis*. Deoxyartemisinin and artemisinic acid did not exhibit any anthelmintic activity against *S. mansoni*. The docking score for ligands towards each protein was calculated to estimate the binding free energy, with the best docking score (lowest energy value) evaluation and *Mt*KasA was suggested as a target for artemisinic acid.

4.1. INTRODUCTION

China is considered as one of the most botanically bio-diverse countries in the world. Out of such biodiversity, over 7000 plant species have medicinal uses and are utilised in Traditional Chinese Medicine (TCM). TCM represents an important corpus of "folk-medicine" than could be seen as the product of centuries of clinical practice approaches used by doctors (Kuhn & Wang, 2008; Xutian, 2012). Recently, modern research ways have been used to detect the molecules, define their mode of action and pharmacokinetic and pharmacodynamics properties. As a result, TCM is benefitting from new knowledge in the use of plant species as well as in the most effective applications of identified products following clinical and toxicity trials (Sucher, 2013).

Artemisia annua L. is native to temperate Asia and is commonly called sweet wormwood. It belongs to the family of Asteraceae and the genus *Artemisia* has over 400 species (Figure 4.1). It is a well-established TCM exemplar, referred locally as *qinghao,* meaning green herb, and is commonly used to treat fever (Figure 4.2) (Youyou Tu, 2011). The plant is known to have anti-malarial (J.-B. Jiang et al., 1982; Kuhn & Wang, 2008), anti-inflammatory, anti-tumour and allelopathic activities (Ho, Peh, Chan, & Wong, 2014b).

Figure 4.1: *Artemisia annua* (left); Dried *Artemisia annua* (right).

The most potent anti-malarial natural product from *A. annua* is artemisinin; a sequiterpene trioxane lactone (Van Agtmael, Eggelte, & Van Boxtel, 1999) that recently was the subject of the 2015 Nobel Prize in medicine. Artemisinin and its semi-synthetic derivatives artesunate, artemether and arteether are now front line anti-malarial drugs (Ho et al., 2014b). Common features of all of these drugs are an endoperoxide bridge and conversion into the active metabolite, dihydroartemisinin in the human body (Ho et al., 2014b). The anti-malarial mechanisms of all of these artemisinin drugs remains to be firmly established but is accepted to be dependent on free-radical generation linked to the breaking of the distinctive endoperoxide bridge by a haem-mediated reaction (Jefford et al., 1996; Meshnick, Thomas, Ranz, Xu, & Pan, 1991). Within malaria causing *Plasmodium* species, the killing mechanisms may include haem and protein alkylation as well as disruption of a sarcoplasmic, endoplasmic

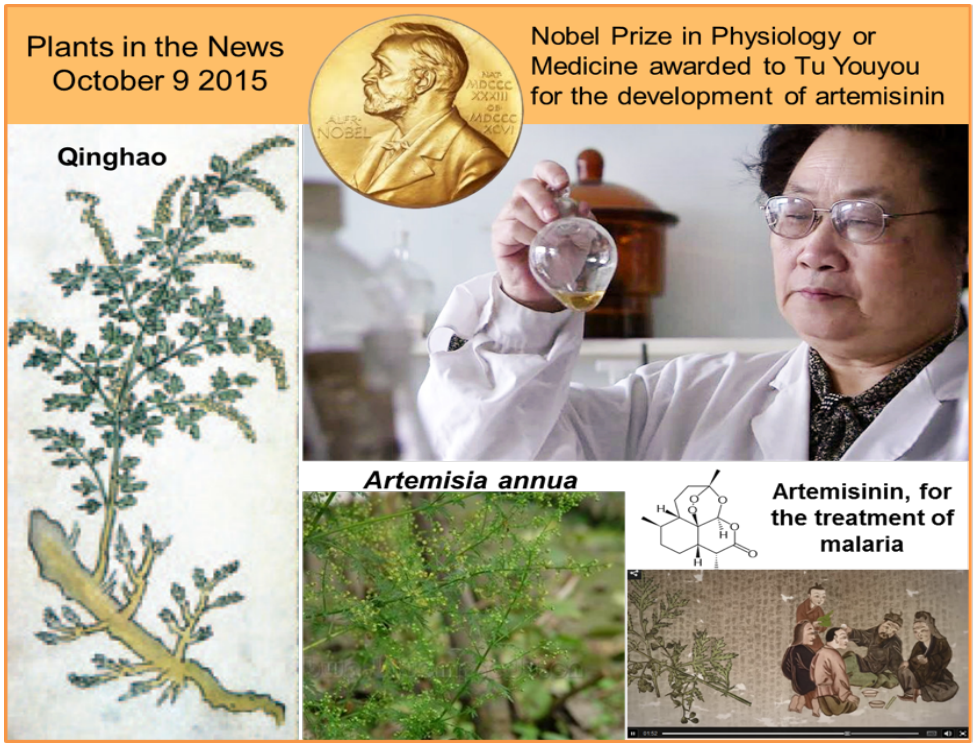


Figure 4.2: Image credit: South China Morning Post.

reticulum PfATPase6 calcium pump (SERCA) (Ding, Beck, & Raso, 2011).

Beyond *Plasmodium*, artemisinin (and its semi-synthetic derivatives) exhibits activity against other protozoan parasites including *Trypanosoma* (Mishina, Krishna, Haynes, & Meade, 2007) and *Toxoplasma* (Chen et al., 2007; Ou-Yang, Krug, Marr, & Berens, 1990) as well as metazoan parasites including blood fluke species of *Schistosoma* (Xiao, You, Yang, & Wang, 1995). Where the mechanism of action has been defined, this is linked to oxidative events. For example, in *Schistosoma mansoni,* artemisinins have been linked to increase lipid peroxidation and reduced glutathione levels (El-Bassiouni et al., 2007; Zhai, Jiao, Mei, & Xiao, 2002). Increased oxidative effects have also been linked to artemisinin's anti-viral activity (Efferth et al., 2008), anti-fungal activity and anti-cancer activity (Kaptein et al., 2006).

Interestingly, the activity of artemisinin against bacteria remains poorly defined. In a mouse model, artesunate was shown to synergise with ampicillin to reduced sepsis linked to infection with *Escherichia coli* (Jun Wang et al., 2006). However, rather than any direct antimicrobial effects, the observed improved mortality rates appeared to be linked to reducing inflammatory effects (Shi, Li, Yang, & Hou, 2015; Jun Wang et al., 2006). Similarly, synergistic improvements are observed with artemisinins administered with other antibiotics to treat *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) infection in association with reduced inflammation (Jiang et al., 2011; Li et al., 2015). Furthermore, while artemisinin itself has minimal anti-tuberculosis activity, when attached to a *Mycobacterium tuberculosis* siderophore shows significant potency (Miller et al., 2011; Ratledge, 2004). Siderophores are bacterial metal chelators that play an important role in iron assimilation and virulence. Siderophore conjugation is likely to improve the anti-tubucular activity of artemisinin by increasing the availability of iron for endoperoxide bridge linked oxidative events and / or easing bacterial penetration by the drug (Miller et al., 2011) (Figure 4.3). Given the wide-ranging activities of artemisinins (Ho et al., 2014b) and anti-mycobacterial properties of conjugated artemisinin, we sought to identify artemisinin-like molecules from *A. annua* that,

by themselves, contain potent anti-microbial activity. Thus, we isolated a small library of compounds with anti-mycobacterial action from *Artemisia annua*. These were identified as deoxyartemisinin and artemisinic acid which, whilst showing some structural similarity to artemisinin, lacked the endoperoxide bridge.

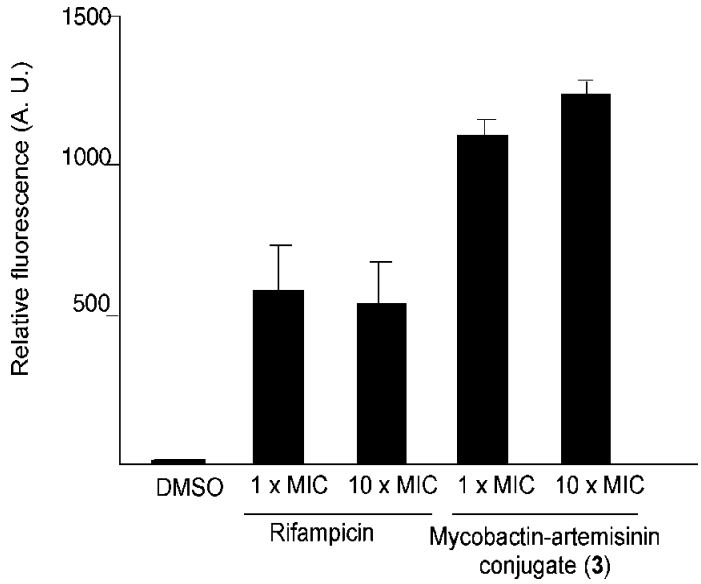


Figure 4.3: *Mycobacterium tuberculosis* siderophore activity.

H37Rv was treated with rifampicin (0.1 µg mL^{-1} and 1 µg mL^{-1}) or mycobactin-artemisinin conjugate 3 (0.5 µg mL^{-1} and 5 µg mL^{-1}) at 1 and 10 MIC. Following 3 h incubation, reactive oxygen species were measured with HPF (Miller et al., 2011).

4.2. METHODS

Extraction and Isolation

Dried leaf material (200 g) of *Artemisia annua* supplied from Chinese sources was extracted sequentially using *n*-hexane, dichloromethane, ethyl acetate and methanol (2×3L) at room temperature with continuous stirring for 24 hours in each case. The fractions were dried and resuspended in 50 % aqueous methanol. These crude extracts were screened for antimicrobial activities and the MIC for each was determined.

Purification

The *n*-hexane extract (18.49 g) fractionated on a silica gel CC (4×50 cm, 150 g of SiO_2), eluted with *n*-hexane-EtOAc (1:0 to 0:1, at 5 % gradient, 300 ml of each eluent), and EtOAc- MeOH (1:0 to 8:1, 300 mL of each eluent) to yield 14 fractions (designated H for "*n*-hexane" and A through to N; thus HA through to HN). Fraction HF (2.86 g) was again subjected to silica CC (1.5×50 cm, 60 g of SiO_2) eluting with *n*-hexane-EtOAc (1:0 to 0:1, 5% gradient, 300 mL of each eluent) and EtOAc-MeOH (1:0 to 8:1, 300 mL of each eluent) to yield 6 crude fractions (A through F; thus HFA-HFF). The HFE fraction (824.7 mg) was then run again fractionated using reversed-phase flash chromatography, eluted with a methanol/water gradient resulting in 7 fractions (HFE1 through HFE7). HFE3 was then subjected to further fractionation in a Semi-preparative HPLC, to yielding 8 fractions (HFE3a through HFE3h). Fraction HFE3e was pure compounds compound 1 (6 mg) and HFE3f was compound 2 (4.5 mg) at > 95% purity.

Synergy assay

The compounds were further screened for their synergistic effect on each other. Compounds were dissolved in MeOH and diluted to a sub inhibitory concentration. The compounds were serially diluted for the MIC determination in triplicate. The fractional inhibitory concentration

index (FICI) expressed the effect of the combination of antibacterial agents (Eliopoulos et al., 1996):

$$FICI = FIC\ (A) + FIC\ (B)$$

$$FIC\ (A) = MIC\ (A \text{ in presence of } B)/MIC\ (A \text{ alone})$$

$$FIC\ (B) = MIC\ (B \text{ in presence of } A)/MIC\ (B \text{ alone})$$

$FICI \leq 0.5$, Synergism; $FICI > 4.0$, Antagonism; $FICI > 0.5$ to 4.0 no interaction.

Anti-Schistosoma assay

The anthelmintic activities of compounds were assessed against the schistosomula lifecycle stage of *Schistosoma mansoni* as previously described (Nur-E-Alam et al. 2017). Briefly, all compounds were solubilized in DMSO (Fisher Scientific, 10122140) and screened in concentrating range from 0.625 to 10 μM against ~120 schistosomula per well, within a 384 well plate (Perkin Elmer, 6007460) in triplicate. Schistosomula were cultured in 80 μL of Basch media (Basch 1981) containing a final DMSO concentration of 0.625 %. To assess the effect of each compound on schistosomula following 72 hours of culture (37 °C, 5 % CO2, humidified environment), the motility and phenotype of schistosomula was assessed based on the image analysis model reported previously (Paveley et al. 2012). All results were tested for significance using one-way ANOVA with a Dunn's post-test to measure significant differences ($P < 0.05$) between DMSO control wells and individual treatments.

***In silico* studies**

All chemical structures were retrieved from the PubChem compound database (NCBI) (http://www.pubchem.ncbi.nlm.nih.gov). The crystal structures and control ligand inhibitors (Table V) of MtDprE1 (PDB ID: 6HEZ), MtInhA (PDB ID: 1ENY), MtKasA (PDB ID: 2WGE), MtPanK type 1 (PDB ID: 4BFT), MtPknB (PDB ID: 2FUM) and MtPks13 (PDB ID:

5V3X) were retrieved from the RCSB Protein Data Bank (PDB) database (https://www.rcsb.org).

In silico prediction of physico-chemical and structural properties of the compounds was performed using PaDEL-Descriptor (Yap 2011) including the descriptors: nHBAcc_Lipinski (acceptor H-bonds), nHBDon_Lipinski (donor H-bonds), nRotB (number of rotation bonds), TopoPSA (topological polar surface area), MW (molecular weight) and XLogP (prediction of logP based on the atom-type method).

An extended PDB format, termed a PDBQT file, was used for coordinate files, which includes atomic partial charges (Umamaheswari et al. 2012). All file conversions were performed using the open source chemical toolbox Open Babel 2.3.2 (O'Boyle et al. 2011). The ligand and protein structures were optimised using AutoDock Tools software (AutoDock 1.5.6) which involved adding all hydrogen atoms to the macromolecule, which is a step necessary for correct calculation of partial atomic charges. Gasteiger charges are calculated for each atom of the macromolecule in AutoDock 1.5.6 (Umamaheswari et al. 2012).

All the compounds were docked against target proteins along with the controls. Molecular docking calculations for all compounds with each of the proteins were performed using AutoDock Vina 1.1.2. Docking calculation was generated with the software free energy binding own scoring function. The binding affinity of the ligand was observed as a negative score with units expressed as $kcal.mol^{-1}$. Nine different poses were calculated for each protein with the parameters num_modes = 9 and exhaustiveness = 16. The lowest energy conformation was chosen for binding model analysis. Molecular interactions between ligand and protein were generated and analysed by LigPlot+ and depicted by PyMOL. PyMOL Molecular Graphics System, Version 2.0 Schrödinger (http://www.pymol.org) was used to prepare figures (Ali et al. 2018). To provide enough space for free movements of the ligands, the grid box was constructed to cover the active sites and was defined using AutoDock 1.5.6.

All the compounds were docked against known anti-*Mycobacterium* targets, MtDprE1, MtInhA, MtKasA, MtPanK, MtPknB,and MtPks13 along with their respective control inhibitors for each drug target.

To validate the accuracy of the docking and to allow a comparison between docking scores, all co-crystallised inhibitory ligands were re-docked into the corresponding protein structures. The grid points for MtDprE1, the grid points were set to 20 × 20 × 20, at a grid center of (x,y,z) 14.99, -20.507, 37.226 with spacing of 1 Å. For MtInhA, the grid points were set to 26 × 24 × 22, at a grid center of (x,y,z) -5.111, 33.222, 13.410 with spacing of 1 Å. For MtKasA, the grid points were set to 20 × 20 × 20, at a grid center of (x,y,z) 38.342, -7.033, 13.410 with spacing of 1 Å. For MtPanK, the grid points were set to 20 × 20 × 20, at a grid center of (x,y,z) -18.742, 13.919, 11.679 with spacing of 1 Å. For MtPknB, the grid points were set to 21 × 20 × 20, at a grid center of (x,y,z) 61.518, 2.429, -25.588 with spacing of 1 Å. For MtPks13 the grid points were set to 16 × 18 × 14, at a grid center of (x,y,z) 3.954, 27.324, 8.499 with spacing of 1 Å.

4.3. RESULTS

Wide-ranging anti-microbial activity was observed in the hexane extract against *Mycobacterium smegmatis* albeit at high MICs (Figure 4.4). *M. smegmatis* is a well-established model species for *M. tuberculosis,* and given the known activity of the artemisinin-siderophore conjugate (Miller et al., 2011; Ratledge, 2004). Bioactivity-linked fractionations of the hexane extract were based on the ability to suppress the growth of *M. smegmatis*.

The *n*-hexane fraction was assayed against *M. smegmatis* and HF displayed the highest anti-mycobacterial activity (Figure 4.4). HF was further purified and screened suggesting that fraction HFE had the highest activity (Figure 4.4). Fractions HFE3 (92.68 mg) and HFE7 (30.55 mg) had the highest anti-mycobacterial activity (Figure 4.4). Fraction HFE3 and HFE7 were run through UHPLC-MS and processed with Xcalibur (Figure 4.5), to detect their

Antimicrobial activity (MIC mg/mL) of different solvent extracts.

Extracts	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Mycobacterium smegmatis*	Methicillin-resistant *Staphylococcus aureus*(MRSA)	*Candida albicans*
n-Hexane	≥1	0.5	0.5	≥1	≥0.5
Dichloromethane	≥1	≥1	≥1	≥1	≥0.5
Methanol	≥1	≥1	≥1	≥1	≥0.5

Antimycobacterial activity (MIC mg/mL) of different purified fractions of hexane extracts

Extracts	*Mycobacterium smegmatis*
HA-HC	Not soluble
HD-HE	0.25
HF	0.125
HG-HJ	0.5
HK	≥1
HL-HM	0.5
HN	≥1

Antimycobacterial activity (MIC mg/mL) of different purified fractions of HF extracts.

Extracts	*Mycobacterium smegmatis*
HFA-HFD	Not soluble
HFE	0.25
HFF	0.5

Antimycobacterial activity (MIC mg/mL) of different purified fractions of HFE extracts.

Extracts	*Mycobacterium smegmatis*
HFE1	0.25
HFE2	0.25
HFE3	0.125
HFE4	0.25
HFE5	0.5
HFE6	0.5
HFE7	0.125

Figure 4.4: Bio-assay guided purification of *A. annua* based on MIC against *M. smegmatis*.

differences of mass ion spectra. However, fraction HFE7 was not further studied as there was many peaks in the ion spectra and very low quantities.

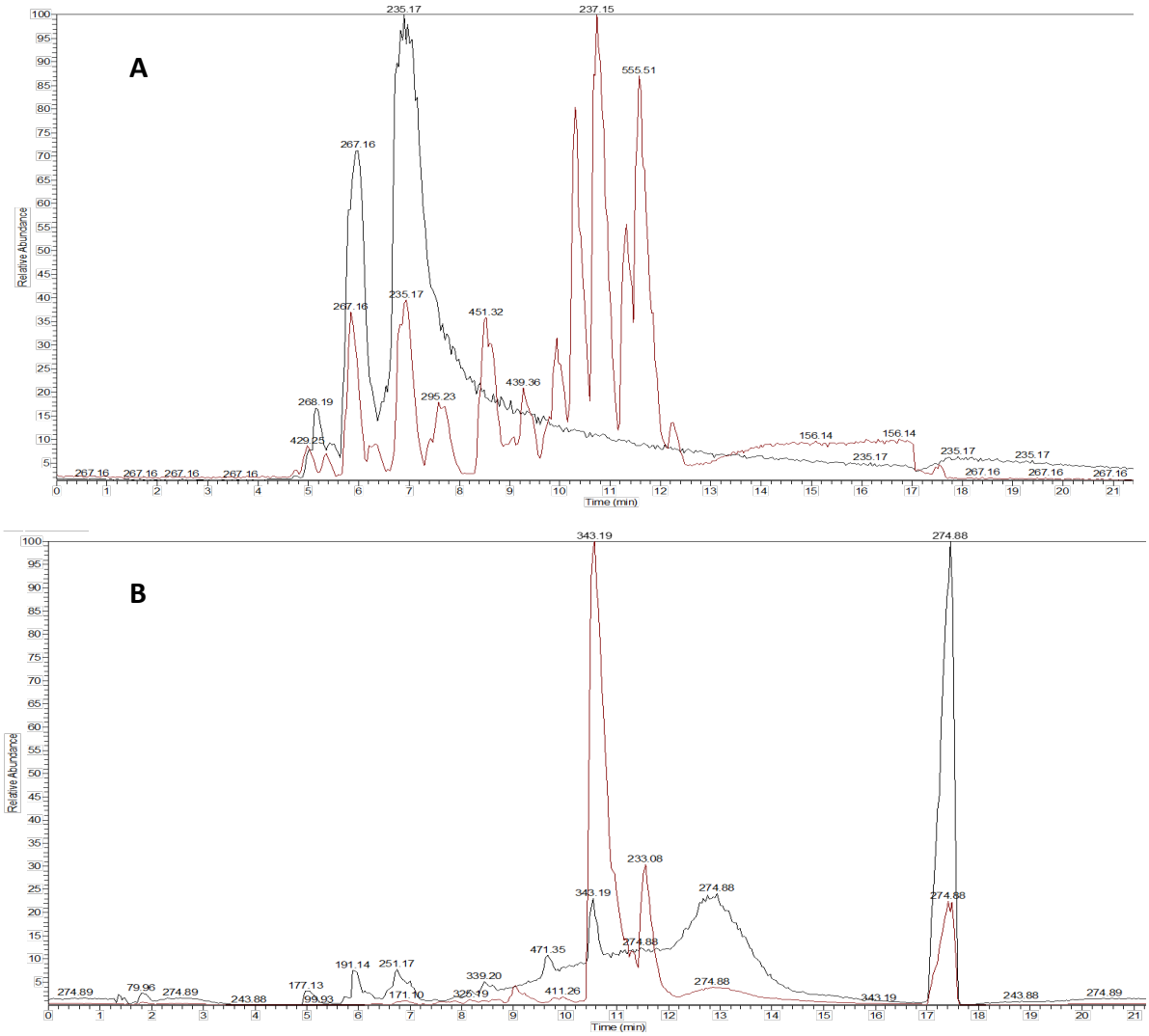


Figure 4.5: Total ion count (TIC) chromatograms of. HFE3(red) and HFE7(black) obtained by UHPLC-MS.

A: Positive Ionisation B: Negative ionisation

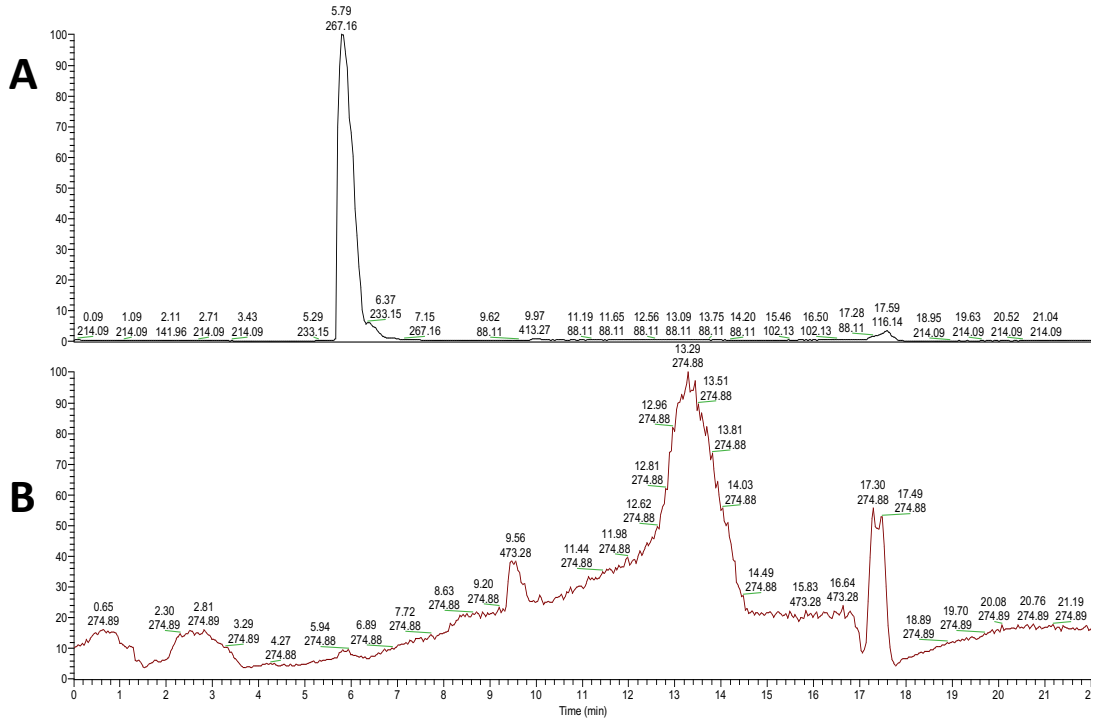


Figure 4.6: Total ion count (TIC) chromatograms of HFE3e.

A: Positive Ionisation B: Negative ionisation

HFE3 was then further fractionated and the fractions were again run through UHPLC-MS (Figure 4.6 and Figure 4.7) to determine the ion mass and semi-preparative HPLC confirming

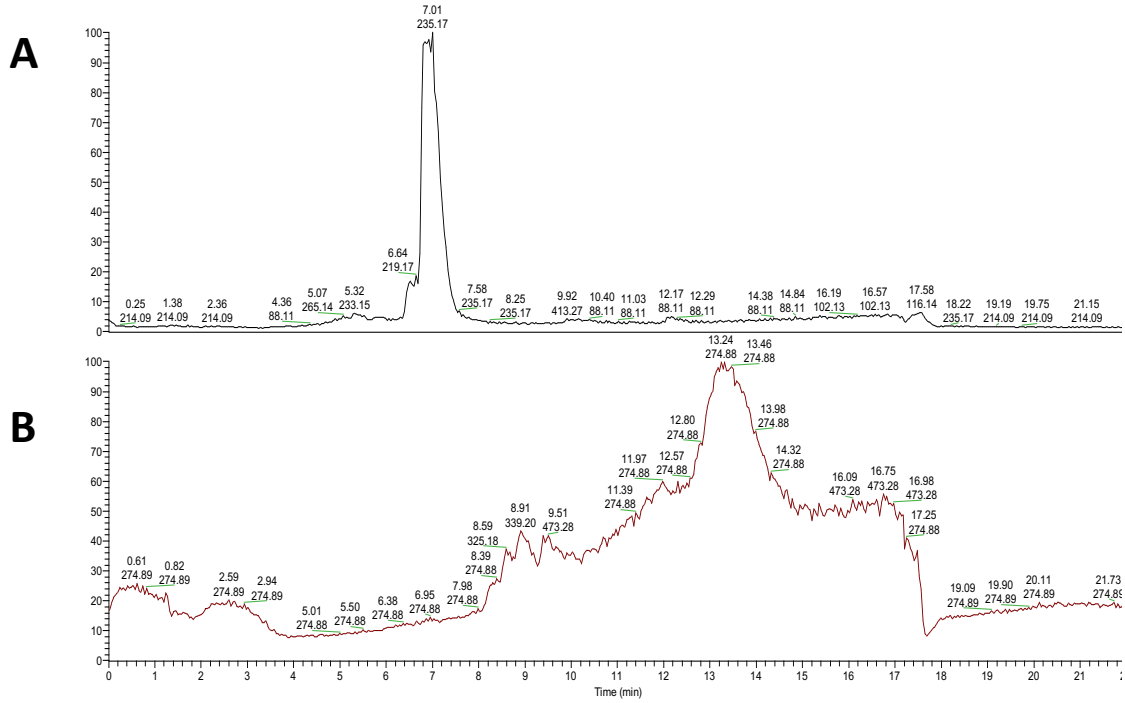


Figure 4.7: Total ion count (TIC) chromatograms of HFE3f.

A: Positive Ionisation B: Negative ionisation

the purity. The identities of the purified compounds 1 and 2 were determined by NMR yielding the following data:

1: ^{1}H-NMR (500MHz, $CDCl_3$) δ 0.93 (3H, s, H-14), 0.99 (2H, m, H-8), 1.11 (1H, m, H-9), 1.19 (3H, d, J=7.2 Hz, H-13), 1.23 (1H, m, H-2), 1.25 (1H, m, H-10), 1.27 (3H, m, H-1), 1.51 (3H, s, H-15), 1.61 (1H, m, H-3), 1.79 (2H, m, H-3' and H-9'), 1.90 (2H, m, H-2' and H-8'), 1.99 (1H, m, H-7), 3.17 (1H, m, H-11), 5.69 (1H, s, H-5) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 12.74 (C-13), 18.69 (C-14), 22.20 (C-2), 23.68 (C-8), 24.09 (C-15), 32.92 (C-11), 33.66 (C-9), 34.17 (C-3), 35.53 (C-10), 42.64 (C-7), 44.85 (C-1), 82.58 (C-6), 99.80 (C-5), 109.32 (C-4), 171.88 (C-12) ppm.

2: ^{1}H-NMR (500 MHz, MeOD) δ 0.92 (3H, d, J = 6.3 Hz, H-14), 1.07 (1H, m, H-9), 1.30-1.45 (2H, m, H-3), 1.40 (3H, m, H-1 and H-8), 1.55 (1H, m, H-10), 1.59 (3H, s, H-15), 1.70-1.83 (2H, m, H-2), 1.90 (1H, m, H-9'), 1.98 (1H, m, H-6), 2.68 (2H, m, H-7), 5.02 (1H, s, H-5), 5.45 (1H, s, H-13), 6.29 (1H, s, H-13') ppm; ^{13}C-NMR (100 MHz, MeOD) δ 20.2 (C-14), 23.8 (C-15), 26.7 (C-2), 27.1 (C-8), 27.3 (C-3), 28.9 (C-10), 36.5 (C-9), 39.4 (C-6), 43.1 (C-1), 43.7 (C-7), 121.5 (C-5), 124.6 (C-13), 135.9 (C-4), 145.3 (C-11), 170.7 (C-12) ppm.

These results indicated that compound 1 was deoxyartemisinin and compound 2 was artemisinic acid. These are known compounds allowing their identification to be confirmed from NMR data in the literature (Bhandari, Gupta, Singh, & Kaul, 2005; Misra, Ahmad, Thakur, Lotter, & Wagner, 1993; Srivastava et al., 2009) and by comparison with commercially obtained standards. In both cases, anti-mycobacterial activity has not been demonstrated for

these natural products. Crucially, both display an artemisinin-like structure but neither possesses the key endoperoxide bridge, which is distinctive for artemisinins. (Figure 4.8)

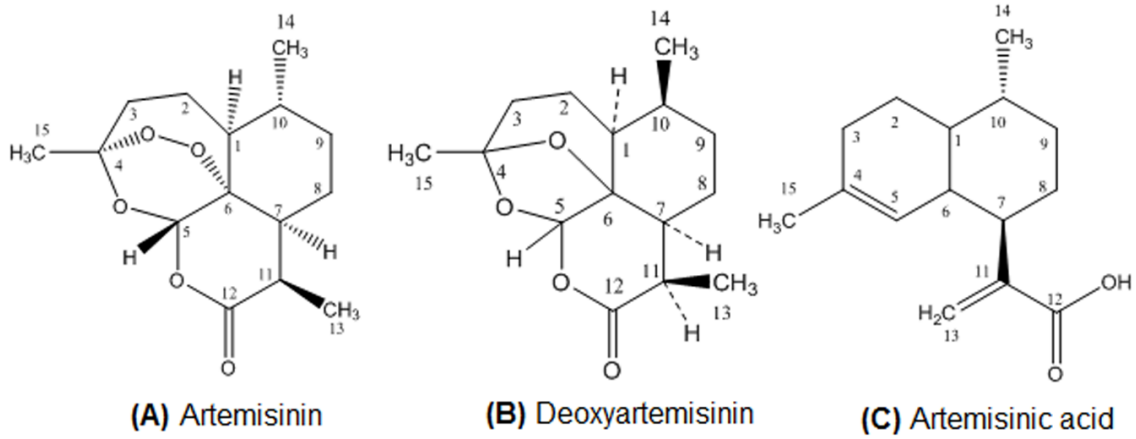


Figure 4.8: The chemical structures of the bioactive natural products isolated from *A. annua.*

B: Deoxyartemisinin, C: Artemisinic acid. A: Artemisinin was used as control to compare the structure and bioactivity

The antimicrobial properties of the compounds 1 and 2 were assessed against our full panel of bacterial strains (Table IV).

Table IV: Antibacterial activity (MIC µg mL^{-1}) of compounds.

Deoxyartemisinin), artemisinic acid and artemisinin against Gram-positive and Gram-negative bacteria

Organisms	Deoxy-artemisinin	Artemisinic acid	Artemisinin	Rifampicin	Gentamycin Sulphate
E. coli	500	≥1000	≥1000	ND	1.6
P.aeruginosa	500	≥1000	≥1000	ND	3.13
S. aureus	500	250	≥1000	ND	3.13
M. smegmatis	500	500	≥1000	1.14	ND
M. smegmatis mc2155	500	250	500	1.41	ND
MRSA	500	250	≥1000	ND	3.13

In isolation, each compound showed some activity against *M. smegmatis,* although less than exhibited by the parental extract (HFE3). Compounds 1 and 2 exhibited some antimicrobial activities, which were significantly better than that shown by artemisinin. We searched for artemisinin in each of fractions based on accurate mass compared to commercially sourced standards, but this was never detected.

The discrepancies between the MICs obtained for the parental fraction (HFE3) against those obtained for the purified compounds could reflect a synergistic action between the two compounds. This was assessed using the checkerboard assay where the two compounds were combined in an anti-mycobacterial screen. We found a synergy effect between the two compounds with FICI=0.5. This demonstrated that the compounds are synergistic and in combination, the MIC for compounds 1 and 2 was reduced by a factor of 4 to 125 μg/mL.

Deoxyartemisinin has been demonstrated to have dramatically reduced anti-malarial activity compared to artemisinin, suggesting that the killing mechanism was dependent on peroxidative activity (Kaiser et al., 2007). In the case of *S. mansoni,* artemisinin, artemisone, dihydroartemisinin and artemether have been shown to be particularly effective, again through the peroxidative activity (Gold et al., 2017). We re-assessed the anti-parasitic properties of deoxyartemisinin and also that of artemisinic acid, which has not been previously assessed. Using the Roboworm platform (Figure 4. 9) (Nur-E-Alam et al., 2017), the impact on the phenotype and motility of *S. mansoni* was measured against those of the front-line anti-*Schistosoma* drug Praziquantel (PZQ) as well as the thioredoxin glutathione reductase (TGR) inhibitor Auranofin (AUR); both of which do not act through peroxidative events (Danso-Appiah, Olliaro, Donegan, Sinclair, & Utzinger, 2013; Kuntz et al., 2007). Using the Roboworm platform, anthelminthic activity of deoxyartemisinin (C1) and arteminisic acid (C2) were compared to those of Auranofin (AUR, 10 μM), Praziquantel (PZQ, 10μM) and the negative control dimethylsulphoxide (DMSO). (A) Images of schistosomula at 72 h following

treatments. Schistosomula treated with AUR and PZQ exhibited obvious phenotypic changes compared to DMSO. Treatments (two-fold dilutions; 10 μM-0.625 μM) with C1 and C2 showed no obvious effect. Viability for (B) C1 and (C) C2 treatments as well as assessments of motility for (D) C1 and (E) C2 were quantified. All results were tested for significance using one-way ANOVA with a Dunn's post-test. Results falling within the "hit zone" were significantly ($P < 0.05$) from negative DMSO treated controls. In no case were significant effects for C1 and C2 observed. Here, neither deoxyartemisinin or artemisinic acid negatively impacted parasite phenotype nor motility when compared to PZQ or AUR.

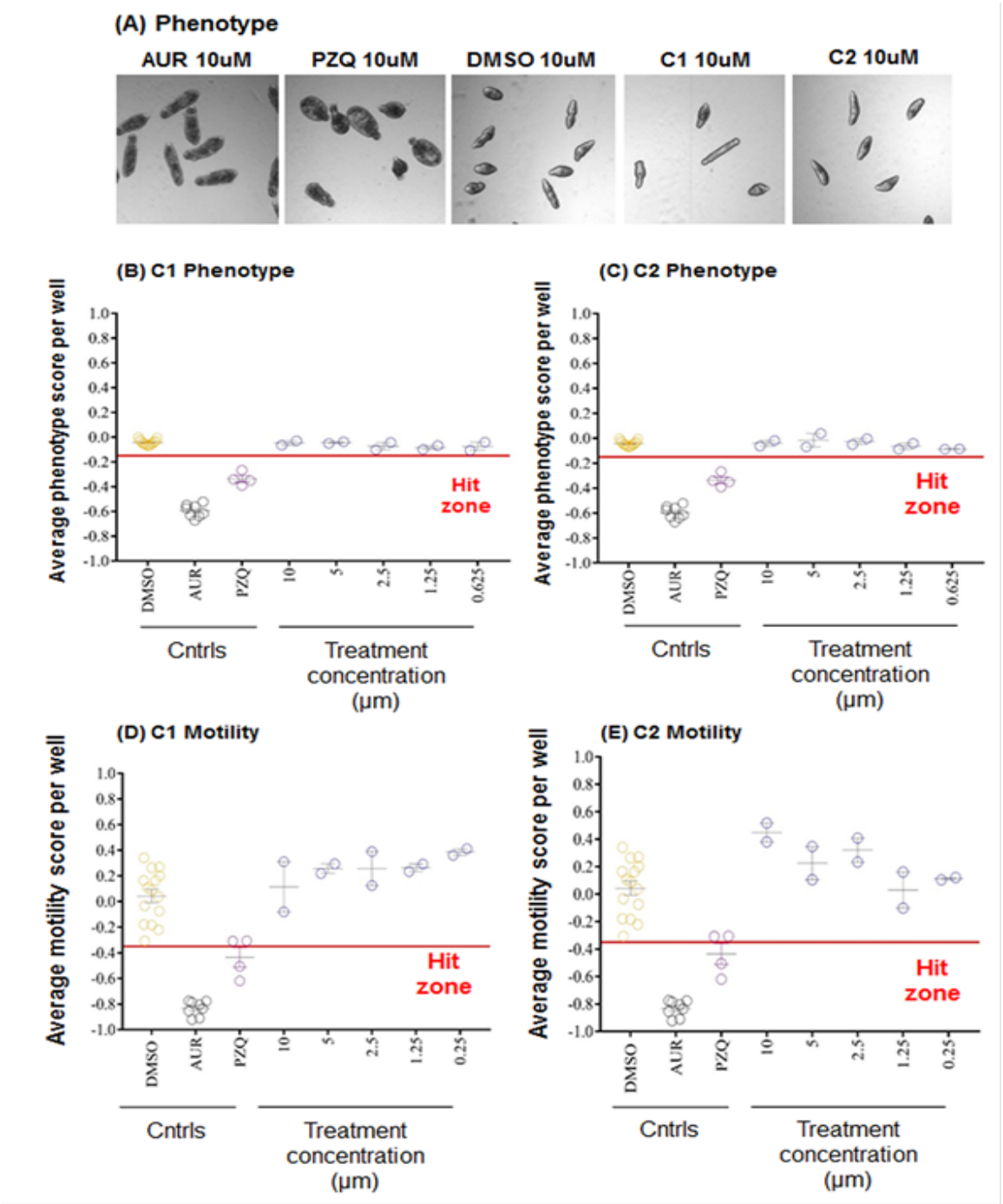


Figure 4. 9: Screening of bioactives isolated for anthelminthic activity against *Schistosoma mansoni* schistosomula.

C1: Deoxyartemisinin, C2: artemisinic acid, AUR: Auranofin; PZQ: Praziquantel

In an attempt to provide further insight into these natural products, their ability to dock to known anti-*Mycobacterium* targets were assessed. Thus, artemisinin, deoxyartemisinin and arteminisic acid were organized in phytochemical groups and their individual binding energies

were analysed by docking against *Mt*DprE1, MtInhA, MtKasA, MtPanK, MtPknB and MtPks13 and compared with their respective control. AutoDock Vina was used to predict binding affinities. Known molecules, that had been reported previously in the literature as inhibitors of the target enzymes and for which the nature and role of the binding site residues were known from their available complexes with the proteins, were used as controls.

Individual binding energy of artemisinin, artemisinic acid and deoxyartemisinin was analysed by docking against MtDprE1, MtInhA, MtKasA, MtPanK, MtPknB and MtPks13, and compared with the respective control inhibitor, retrieved from Protein Data Bank (PDB). AutoDock Vina was used to predict binding affinities (Table V).

Table V: Binding energies (kcal.mol^{-1}) of compounds and their respective controls

Targets	Artemisininc acid	Artemisinin	Deoxy-Artemisinin	Respective controls
MtPks13	-6.7	-7.6	-8.2	I28 (ethyl 5-hydroxy-4-[(4-methylpiperidin-1-yl)methyl]-2-phenyl-1-benzofuran-3-carboxylate): -10.5
MtPknB	-6.7	-8.4	-8	Mitoxantrone: -7.7
MtPanK	-7.6	-8.7	-8.7	ZVT (2-chloro-N-[1-(5-{[2-(4-fluorophenoxy) ethyl] sulfanyl}-4-methyl-4H-1,2,4-triazol-3-yl) ethyl] benzamide): -8.3
MtKasA	-8.3	-6.4	-6.9	Thiolactomycin: -6.57
MtInhA	-8	-8.9	-8.5	Isoniazid: -10.4
MtDprE1	-7.2	-8.3	-8.3	Bedaquiline: -10.1

Artemisinic acid shows lower binding energy against MtKasA in comparison to artemisinin and deoxyartemisinin and also the respective control thiolactomycin (Table V). To further investigate the key properties necessary for an optimal binding, the chemical space spanned by the compounds was studied for all the compounds. The physiochemical properties were studied

using PaDel-Descriptor including: molecular weight (MW), partition coefficient (xLogP), rotatable bonds (nRotB), H-bond donors (nHBDon_Lipinski), H-bond acceptors (nHBAcc_Lipinski) and topological polar surface area (TopoPSA) (Table VI).

Table VI: Physiochemical properties of isolated compounds, artemisinin and thiolactomycin analyzed by PaDel-Descriptor

Name	nHBAcc	nHBAcc2	nHBAcc3	nHBAcc_Lipinski	nHBDon	nHBDon_Lipinski	nRotB	RotBFrac	nRotBt	RotBtFrac	LipinskiFailures	TopoPSA	MW	AMW	XLogP
Artemisinic acid	2	2	2	2	1	1	2	0.111111	5	0.277778	0	37.3	234.162	6.004153	4.592
Artemisinin	5	5	5	5	0	0	0	0	3	0.130435	0	53.99	282.1467	6.717779	3.039
Deoxyartemisinin	4	4	4	4	0	0	0	0	3	0.136364	0	44.76	266.1518	6.491508	2.913
Thiolactomycin	2	2	2	2	1	1	2	0.142857	6	0.428571	0	62.6	210.0715	7.502552	2.458

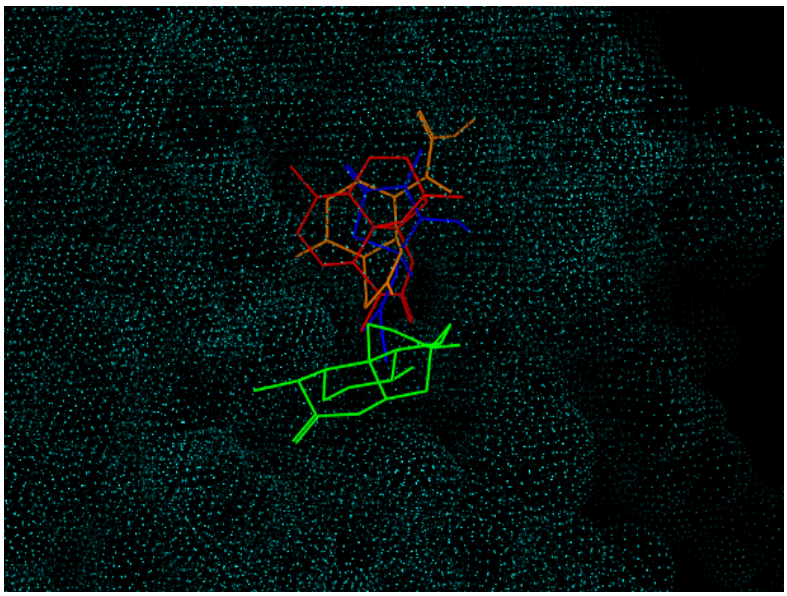


Figure 4. 11: Superposition of the best docking positions of deoxyartemisinin (Red), ng

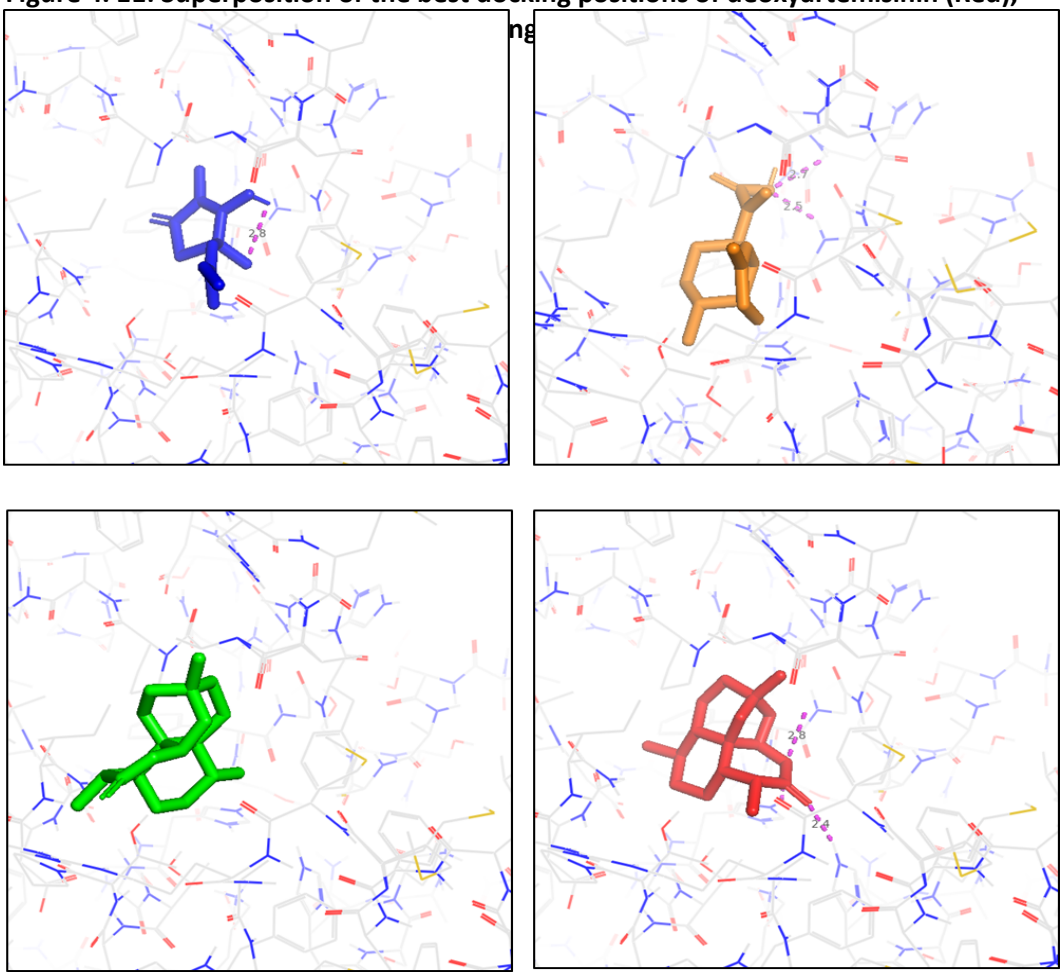


Figure 4. 10: Molecular interactions of the best docking position.

Thiolactomycin (blue: top left), artemisinic acid (Orange: top right) artemisinin (green: down left) and deoxyartemisinin (down right) against MtKasA. Hydrogen bonds are evidenced with magenta dashed lines.

After analysing the binding energies and physiochemical properties, a structural study was carried out based on their binding energy by docking the compounds onto MtKasA and their interaction were further investigated (Figure 4. 10, Figure 4. 11 and Figure 4.12).

A closer look at the interactions between artemisinic acid and MtKasA reveals that the C-12 carboxylic acid end of the molecule have a strong hydrogen bond with nitrogen (contact distance 3.0 Å) and oxygen (contact distance 2.77 Å) of Gly403 and oxygen (contact distance 3.25 Å) Asn 408. While the 4,7-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalen-1-yl group binds to the hydrophobic pocket of Pro280, Phe 402 and His311. The active site, and amino acid are in close similarity to what has been previously described as the mode of binding of the TLM control (Luckner, Machutta, Tonge, & Kisker, 2009a).

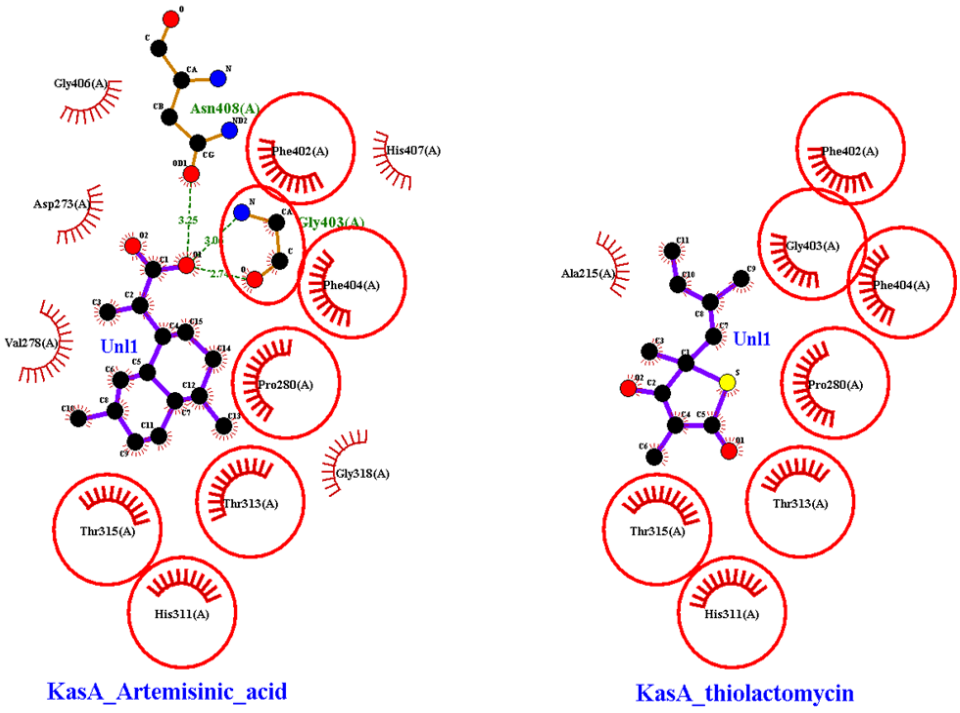


Figure 4.12: Ligplot illustration of Artemisinic acid and thiolactomycin with MtKasA.

H-bonds in green dash. Carbons are in black, nitrogens in blue and oxygens in red. Red spheres are hydrophobic interactions

4.4. DISCUSSION

Artemisinin is a sesquiterpene lactone that is representative of a diverse range of natural products that can be found in the Asteraceae family. Artemisinin has a low aqueous solubility of 63 mg/L at pH 7.2, a Log P of 2.94 (Haynes et al., 2006), and short half-life. The mean oral clearance was 417 L/h, and the terminal elimination half-life was 1.93 h (Birgersson et al., 2016). The molecule releases reactive oxygen species when in contact with high iron concentrations (Gharib, Faezizadeh, Mesbah-Namin, & Saravani, 2014). To overcome these limits, several semi-synthetic and synthetic derivatives of artemisinin are currently being developed or are actively being used to treat malaria, primarily as part of drug combination therapies (Borstnik, Paik, Shapiro, & Posner, 2002; Nosten & Brasseur, 2002). The World Health Organization is now urging the adoption of ACT (Artemisinin Combination Therapy) in which artemisinin is combined with another drug like mefloquine, to reduce the development of artemisinin resistance against the malaria parasite, *Plasmodium falciparum* (Gu, Li, Melendez, & Weina, 2008; Krishna, Woodrow, Staines, Haynes, & Mercereau-Puijalon, 2006). While the precise mechanism of anti-malarial action by artemisinin remains unclear and controversial, the importance of an artemisinic endoperoxide bridge is irrefutable (Jefford et al., 1996; S. R. Meshnick et al., 2003; Steven Meshnick, 2002; Olliaro, 2001; Posner et al., 2001). Much evidence points to Fe (II)-catalyzed reductive cleavage of the endoperoxide bridge within the parasite, leading to an unstable carbon-centered radical capable of forming protein adducts (Steven Meshnick, 2002). An alternative mechanism suggests that heterolytic opening of the endoperoxide moiety of artemisinin yields a hydroperoxide, leading to a reactive carbonium ion intermediate as well as the formation of hydroxyl radicals (Krishna et al., 2006). This pathway would also promote alkylation of protein nucleophiles by structurally different intermediates based on carbon-centered free radical derivatives (Olliaro, 2001).

Along with malaria, TB is one of the most challenging global public health threats confronting humanity with nearly 1 million people currently suffering from infection with the causative agent, *M. tuberculosis*. The development and spread of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* have stimulated global research efforts (Dorman & Chaisson, 2007). As a consequence, the pipeline of potential new drugs has expanded (Stover et al., 2000). In this context, the possible anti-mycobacterial activities of artemisinins are relevant but their wide-spread use as TB drugs would potentially accelerate the selection of drug-resistant *P. falciparum* strains due to the overlapping ranges of both infectious organisms. The antibacterial and antifungal activities of different *Artemisia* species have been reported (Ivanescu, Miron, & Corciova, 2015) but our research strategy here was to assess if *Artemisia* could contain novel antimicrobials.

In a systematic assessment of extracts from dried *Artemisia annua*, only the crude hexane extract of the plant showed activity against gram positive bacterial species (Figure 4.4). Following activity-based purification, two main components (compound 1 - deoxyartemisinin; compound 2 - artemisinic acid) in this fraction of the extract were isolated, purified and identified (Figure 4.8). Neither proved to be artemisinin but both were artemisinin-like molecules. We note that under certain conditions artemisinins are liable for example in water they can to react with the ferrous ion and haem-Fe(II) or in solvents such as dimethyl sulfoxide (DMSO) and degrade very quickly (Haynes et al., 2007) Therefore, we were concerned that we were observing break down products from other artemisinin. However, our extraction conditions using hexane have been not linked to the decomposition of arteminsins. Further, our chemical standards proved to be stable under our chromatographic and biological assay conditions. Therefore, we suggest that the isolated arteminsins represent *bone fide* natural arteminsins from *Artemisia annua.*

The deoxyartemisinin that was isolated from *A. annua* L has an ether bridge instead of an endoperoxide bridge and is inactive against malarial parasites (Klayman, 1985). Previous antimicrobial studies on *S. aureus* with deoxyartemisinin and artemisinin showed a MIC of 1 and 2 mg/ml, respectively (Srivastava et al., 2009). There have been no such anti-mycobacterial studies on deoxyartemisinin as it is often used as a negative control in experiment where artemisinins are assessed for anti-malarial activity. Artemisinic acid was also isolated from *A. annua* L, and the absolute configuration of the structure was deduced previously (Pang et al., 1997). Artemisinic acid has been reported to be a less efficient antimalarial than artemisinin (Ji, Sun, Wang, Yang, & Tu, 2008), although some antibacterial activity on gram positive bacteria (Bhakuni, Jain, Sharma, & Kumar, 2001) but no antifungal activity (Galal, Ross, Jacob, & ElSohly, 2005) has been reported. However, the activity of *A. annua* against *Mycobacterium* has not been previously studied. Both deoxyartemisinin and artemisinic acid exhibited a minimum inhibitory concentration of 0.5 mg mL^{-1}. We noted that these MICs were higher than those reported for these compounds against other bacterial species (Srivastava et al., 2009). This was case, even though the bacterial species that we used are well-established models for anti-microbial studies. Such results could reflect the strains that we used and/or our culture conditions. However, crucially, this MIC fell four-fold in assessments for synergistic effects. Given the common use of artemisinin and its derivatives as combination therapies for anti-malarial treatments, a similar strategy is suggested from this observation.

One important observation was that the anti-mycobacterial activity of both *Artemisia*-derived natural products was not dependent on an endoperoxide bridge. Similarly, the activity of both molecules could not be linked to an α-methylene-γ-lactone group in natural products from the *Artemisia* genus which has been linked to a range of biological activities including antimicrobial (Ivanescu et al., 2015). Our data would therefore suggest a discrete mode of action for the *Artemisia*-isolated natural products reported herein. Given the structural

similarity of deoxyartemisinin to artemisinin, this previously unsuspected mode of anti-mycobacterial action could also be exhibited by the wider artemisinin class of drugs.

We sought to demonstrate that these two compounds were not active against co-endemic parasites, thereby offering the possibility of specific anti-mycobacterial activity. To begin to assess this, we noted that in addition to anti-malarial properties artemisinin derivatives also have anti-schistosomal properties. For example, artemether (Xiao, Booth, & Tanner, 2000) and artesunate (Batty, Ilett, Timothy, & Davis, 1996) have shown prominent anti-schistosomal activity. However, neither deoxyartemisinin nor artemisinic acid exhibited any effect on *S. mansoni* viability or mobility. Thus, we have no evidence that the anti-mycobacterial mode of action for these natural products is relevant to helminth parasites. Whether these compounds are active against *P. falciparum* is currently unknown.

Furthermore, we investigated the anti TB potential of the compounds using a guided molecular docking approach to find a potential target. We selected some key enzymes required for *M. tuberculosis* to grow and survive within the eukaryotic host, that were involved in essential mycobacterial pathways and were absent from mammalian cells. Thus, predicted binding affinities of isolated *A. annua* compounds against seven *M. tuberculosis* enzymes - caseinolytic peptidase P (MtClpP1P2) (Schmitza, Carneyb, Sellob, & Sauera, 2014), decaprenylphosphoryl-β-D-ribose 2′-epimerase 1(MtDprE1) (Batt et al., 2012; Richter et al., 2018), Enoyl-ACP Reductase (MtInhA) (Dessen, Quemard, Blanchard, Jacobs, & Sacchettini, 1995; Rozwarski, Vilcheze, Sugantino, Bittman, & Sacchettini, 1999), β-ketoacyl acyl carrier protein synthase I(MtKasA) (Luckner, Machutta, Tonge, & Kisker, 2009b), pantothenate kinase (MtPanK) (Bjorkelid et al., 2013), protein kinase B(MtPknB) (Wehenkel et al., 2006) and polyketide synthase (MtPks13) (Aggarwal et al., 2017; Gavalda et al., 2014). Henceforth, we performed molecular docking and predicted interactions, using AutoDock Vina (Trott & Olson, 2010). Molecular docking is widely used to model interactions at the atomic level

between a small molecule (ligand) and a known macromolecule. Several studies have successfully used AutoDock Vina to investigate the interactions of natural products against specific protein targets, including mycobacterial enzymes (Meng, Zhang, Mezei, & Cui, 2012; Sundarrajan, Lulu, & Arumugam, 2015; Yadav et al., 2013).

The purpose of molecular docking is to use scoring algorithms to estimate the likelihood of the compound to bind to the protein ligand. Based on our MIC results and binding energies, it is likely that the artemisinic acid displayed a strong predicted binding with the *Mt*KasA enzyme in comparison to artemisinin, deoxyartemisinin and its control thiolactomycin. Figure 4. 11 and Figure 4. 10 indicates that all the compounds accept artemisinin enter the binding site of thiolactomycin and artemisinic acid forms stronger hydrogen bonding in comparison to deoxyartemisinin acid and thiolactomycin. The docking studies provided strong evidence that the molecular basis for this activity is probably due to KasA inhibition.

Mycolic acids are unique to mycobacteria and play a crucial role in the architecture of the mycobacterial cell envelope. Fatty acid biosynthesis involves two synthases, namely, type I fatty acid synthase (FAS-I) and type II fatty acid synthase (FAS-II). KasA, the mycobacterial β-ketoacyl ACP synthase I is an essential enzyme in the mycobacterial fatty acid biosynthesis (FAS-II) pathway (Figure 4.13) (Kremer et al., 2002) (Lu, Zhang, & Rock, 2004). This enzyme

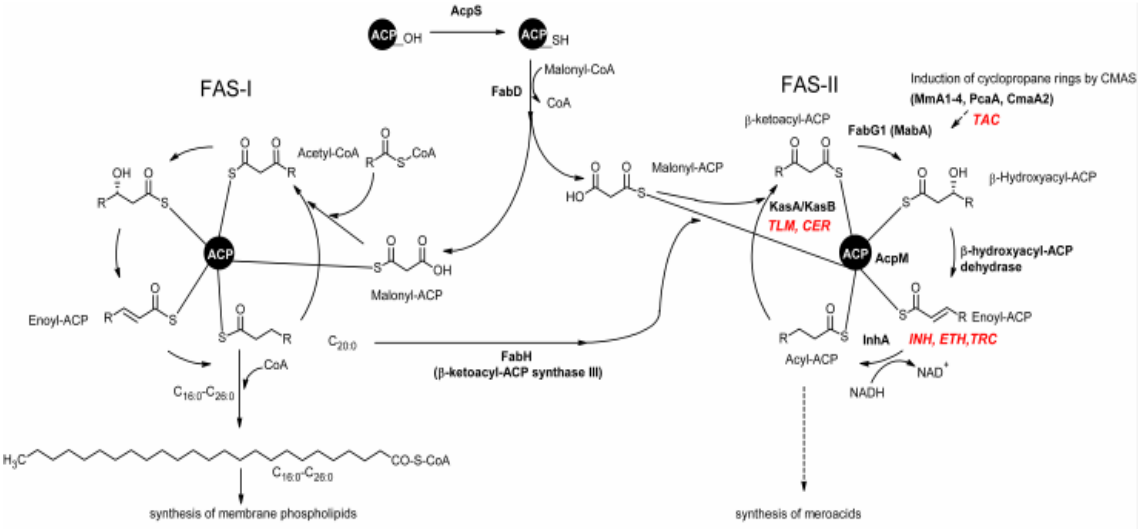


Figure 4.13: Fatty acid biosynthesis with antibiotics and its known target points.

is hypothesized to be a potential target yet to be explored (Bhatt, Kremer, Dai, Sacchettini, & Jacobs, 2005).

Molecular docking technology is the main method of computer-aided drug design (CADD). With the rapid rise in genomics, proteomics, metabolomics, and other omics technologies, as well as the mutual integration of various subject areas, this technology can help to explore the mechanisms of action for treating diseases and guidance the development of natural products to potential drug-leads. However, there are still some common problems in molecular docking which impact on its utility as an approach. There are still differences between the virtual data obtained by molecular docking and the experimental data *in vivo*, and it needs to be verified in combination with other experimental methods. This will also filter out any spurious interactions with non-biological relevance. Validation is also important as the existing evaluation methods for molecular docking technology are still immature, and molecules with higher scores may not be the best ligands. Further, its success is dependent on existing protein structures held in databases so these need to be constantly updated. It is also the case, that existing molecular docking software programs are cumbersome and complex, and they need to be optimized (Chen, Seukep, & Guo, 2020). Therefore, this hypothesis needs to more carefully investigated and validated *in vitro.* This could involve cloning and expressing the putative target so that interaction can be directly assessed as well as the impact on protein function. This approach can be complemented by genomic approaches involving such as transcriptomics and metabolomics where the impact of the natural product on the presumed target can be described.

4.5. CONCLUSION

Our studies have demonstrated that, even in a species which is a well-characterised sources of natural products, novel bioactivities can be defined. Our definition of anti-mycobacterial activities in natural products from *A. annua* was particularly relevant to artemisinin research.

It indicated endoperoxide bridge-independent activity that was exhibited at least against *Mycobacterium*. A virtual screening of the compounds was performed, which indicated that KasA could be a potential target of artemisinic acid and is responsible of its MIC. The interaction of KasA and the compounds needs to verified on the basis of efficacy; toxicity and pharmacokinetic properties

5

Identifying anti-microbials in *Dryopteris crassirhizoma*

Abstract

Dryopteris crassirhizoma Nakai, is a perennial herbaceous fern, is listed in Chinese Pharmacopoeia as a treatment of viral diseases. It is also known to have anticancer, antioxidant, antibacterial, anti-inflammatory, antimalarial and antitumor activities.

Work in this chapter aimed to explore different methods to isolate and purify bioactives from *D. crassirhizoma* including a range of screens focused on anti-microbial activities. Part I: comprises of bioassays coupled with a successive series of chromatographic separation to identify some key chemicals; Part II: Chemometric approaches to profile active and non-active fractions and identify bioactive metabolites, after a round of fractionation, using flow infusion electrospray – high resolution mass spectrometry (FIE-HRMS). Initially screens identified significant activity in *D. crassirhizoma* extracts against Methicillin resistant Staphylococcus aureus (MRSA). The minimum inhibitory concentrations (MIC) of different extract fractions ranged from 3-50 µg/mL.

Bioassay guided of HB sub-fractions resulted in fractions HB5d/e3 and HB5d/e5 with MICs of 6.125 and 12.25 µg/mL respectively. The targeted key metabolites were identified and confirmed by UHPLC-MS and tandem mass spectroscopy (MS2/MS3) data as norflavaspidic acid AB and flavaspidic acid AB. While, chemometric approach on HL sub-fraction the second most active and comparison with the literature, three compounds, flavaspidic acid AB, filixic acid ABP and dryocrassin ABBA were predicted to have antimicrobial activity against *S. aureus* and MRSA. Further purification using preparative-HPLC and tests are required for full structural elucidation and bioctivity.

5.1. INTRODUCTION

Dryopteris crassirhizoma Nakai, a perennial herbaceous fern (Figure 5.1), known as "the king of antivirals", is widely distributed in Korea, China and Japan (Gao et al., 2008). The roots, known as "Gwanjung" in Korea; "Guan Zhong" in China and "Oshida" in Japan, are used in TCM to treat parasitic infestation, haemorrhage, epidemic flu, cold, and cancer (Committee, 1999; *Encycl. Ref. Tradit. Chinese Med.*, 2003; C. Lu et al., 2012; Shinozaki, Shibuya, Masuda, & Ebizuka, 2008). Powdered and dried rhizomes of various *Dryopteris* ferns have been used as remedies for helminthiasis caused by *Diphyllobothrium latum* (Murakami & Tanaka, 1988). Previously reported phytochemical constituents include triterpene, phloroglucinol, flavonoids and other phenolic compounds (Chang, Li, Koike, Wu, & Nikaido, 2006; Min, Tomiyama, Ma, Nakamura, & Hattori, 2001; Noro et al., 1973; Shiojima, Arai, & Ageta, 1990).

Several triterpenes isolated from the dried and fresh leaflets of *D. crassirhizoma,* with hopane

Figure 5.1: Dryopteris crassirhizoma Nakai.

Image credit: Shaanxi Yongyuan Bio-Tech Co.Ltd.

or migrated hopane skeletons (25) (Ageta, Shiojima, Arai, Kasama, & Kajii, 1975; Hiroyuki Ageta, Iwata, & Natori, 1963; Shiojima, Suzuki, Matsumura, & Ageta, 1994). Dryopteric acids A (26) and B (27) were isolated from *D. crassirhizoma* rhizome and showed potent inhibitory

activities against HIV-1 protease with IC_{50} values 26.5 and 44.5 μM respectively (Lee et al., 2008) (Figure 5.2).

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure. They are ubiquitously present in plants and are synthesized through the phenylpropanoid pathway (Waterman, 1986) and occur as aglycones, glycosides, and methylated derivatives. A range of kaempferol glycosides which are inhibitors of human immunodeficiency virus-1 (HIV-1) reverse transcriptase have been isolated from the rhizome of *D. crassirhizoma.* These were designated crassirhizomosides A, B, C and sutchuenoside A (Min et al., 2001) (Figure 5.3).

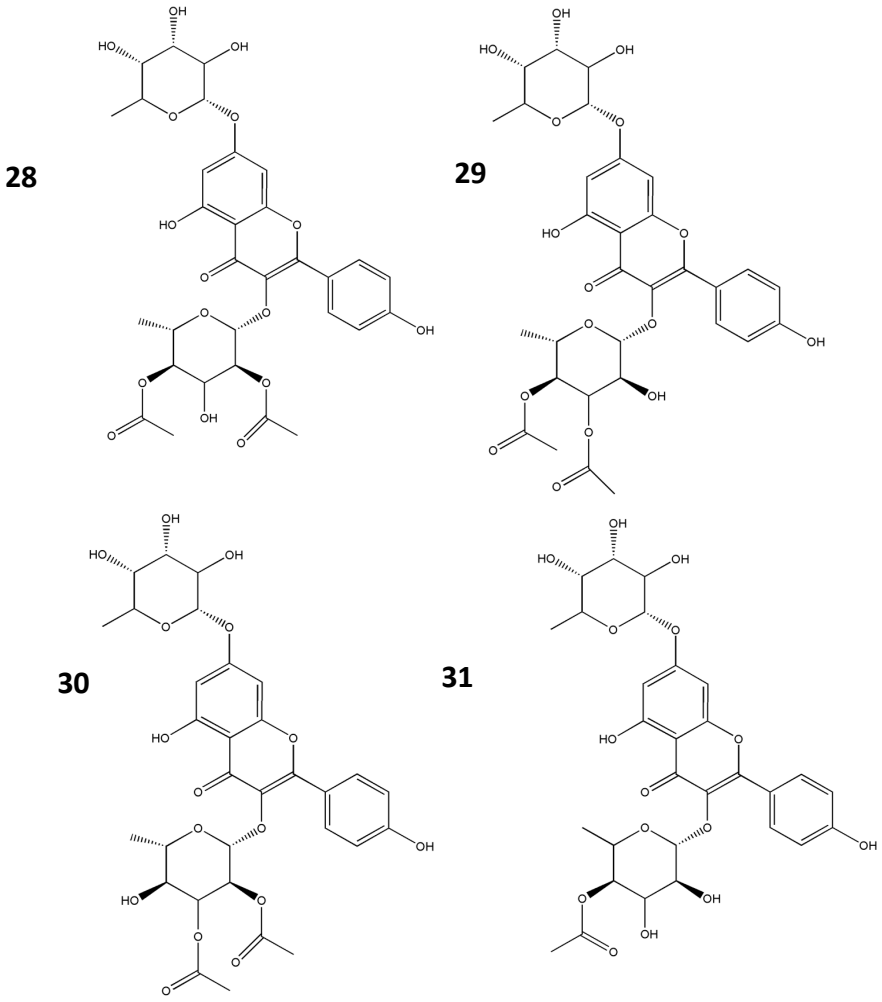


Figure 5.2: Some known flavonoids isolated from *D.crassirhizoma*.

28: Crassirhizomoside A; 29: Crassirhizomoside B; 30: Ccrassirhizomoside C; 31 Sutchuenoside A

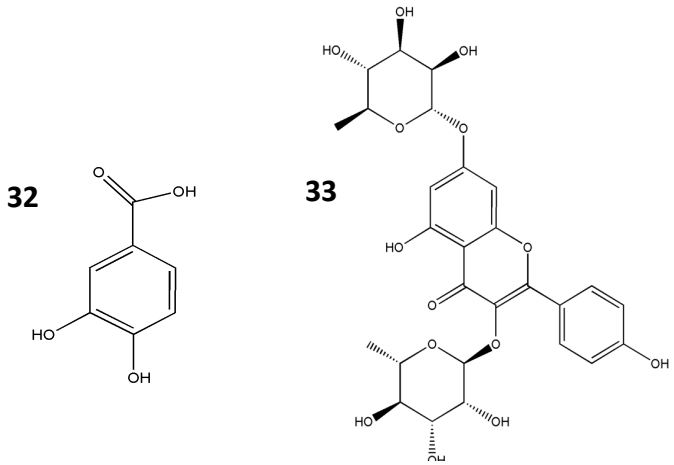


Figure 5.3: Some polyphenols isolated from *D.crassirhizoma*.

32: Protocatechuic acid;33: Kaempferitrin

Phloroglucinol derivatives are a major class of secondary metabolites of wide occurrence in the Myrtaceae family as well as in several other families such as Guttiferae, Euphorbiaceae, Aspidiaceae, Compositae, Rutaceae, Rosaceae, Clusiaceae, Lauraceae, Crassulaceae, Cannabinaceae and Fagaceae. These compounds have also been reported to occur in marine and microbial sources. Phloroglucinol is an organic compound used in the synthesis of pharmaceuticals and explosives. Phloroglucinol, and its benzenetriol isomers, are also defined as "phenols" according to the IUPAC official nomenclature rules of chemical compounds. Such chemicals are often termed "polyphenols" by the cosmetic and parapharmaceutical industries, but this not a scientifically accepted definition. *D. crassirhizoma* also contains the acyl phloroglucinol derivatives. They exhibit a wide range of bioactivity including antimalarial, antibacterial, antiviral, antidepressant, anti-allergic, anti-inflammatory, antifeedant, antidiabetic, algicidal potential. phloroglucinols have not been fully explored for their biological activity, and for many compounds, only the structures have been elucidated (Pal Singh & Bharate, 2006). Phloroglucinol derivatives albaspidin, aspidin, flavaspidic acids, dryocrassin ABBA, filixic acid (Hisada & Noro, 1961), flavaspidic Acid PB and AB (Lee, Na, Na, Min, & Lee, 2003) were isolated from this plant species. Polyphenols like protocatechuic

acid and kaempferitrin were also isolated from the rhizome (Jiang, Chi, Fu, Zhang, & Wang, 2013) (Figure 5.3).

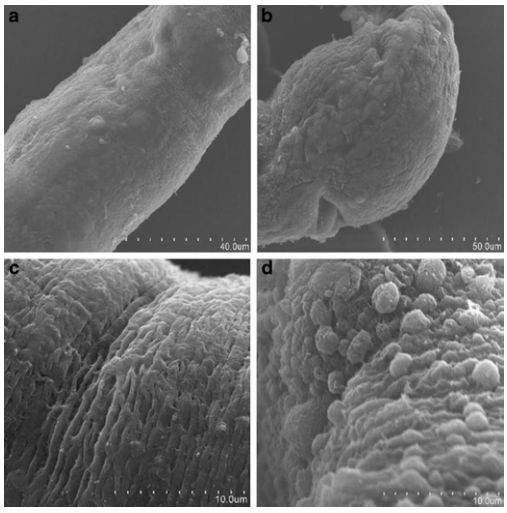


Figure 5.4: *Dactylogyrus intermedius* scanning electron micrographs.

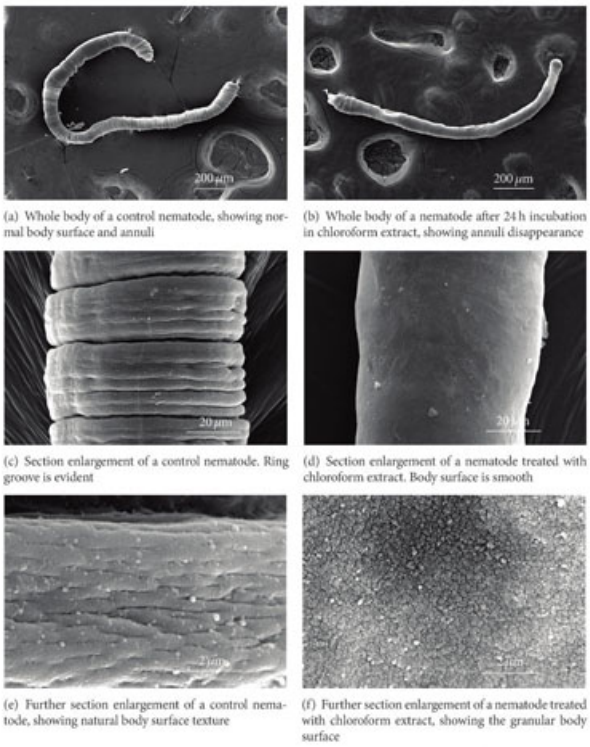
A:Untreated control helminth, showing smooth surface (scale bar = 40 µm); B: Distinct contraction of body surface (scale bar = 50 µm), C: view of ventral surface showing abnormal tegumental folds (scale bar = 10 µm), and D: clumps of dishevelled protuberances (scale bar = 10 µm). Image credits: Jiang et al. 2013

Several components from *D. crassirhizoma* have been reported to have antiviral activity**.** Crassirhizomosides A, B, C and sutchuenoside A are HIV-1 reverse transcriptase inhibitors (Min et al., 2001) inhibitory activity against HIV-1 protease (Lee et al., 2008). Dryocrassin ABBA may have the potential to be used against influenza virus H5N1 infection (Wang et al. 2017). A series of acylphloroglucinols has been reported to have inhibit fatty acid synthase with IC50 values ranging from 23.1 ± 1.4 to 71.7 ± 3.9 µM (Na et al., 2006). *D. crassirhizoma* has also shown to have anti-cancer properties by inducing cell cycle arrest and apoptosis through the extrinsic and intrinsic pathway in PC3-MM2 cells (Chang et al., 2010). Two chemicals from *D. crassirhizoma*, flavaspidic Acid PB and flavaspidic Acid AB have also shown antioxidant activity (Lee, Na, Na, Min, & Lee, 2003). An acidic polysaccharide fraction (DCP-3) was obtained from *D. crassirhizoma* have also been reported to have a strong activity for scavenging DPPH radical (IC50: 2.04 mg/mL), hydroxyl radical (IC50: 1.70 mg/mL), and superoxide anions (IC50: 4.20 mg/mL) and also was capable of reducing ferric ions (Zhao et

al., 2019). Sutchuenoside A and kaempferitrin were observed to be effective against *D. intermedius* with median effective concentration (EC50) of 3.01 and 2.71 mg L^{-1}, respectively (Jiang et al. 2013) (Figure 5.5).

D. crassirhizoma also known to be used for verminosis was investigated for anti-parasitic properties. Phloroglucinol compounds like aspidin, flavaspidic acid, methylene-*bis*-aspidinol and desaspidin were incubated with *Schistosoma mansoni* adult worms for 24 h. Aspidin and flavaspidic acid showed decrease motor activity with tegumental alterations while methylene-bis-aspidinol and desaspidin showed decrease motor activity without tegumental alterations (Magalhães et al. 2010). The chloroform extract of *D. crassirhizoma* demonstrated ultrastructural changes in *M. incognita* after treatment at 1 mg·mL^{-1} for 24 h (Liu, Xie, Feng, & Cai, 2013) (Figure 5.6)

Flavaspidic acid PB and AB showed antibacterial activity on a range of bacteria with MIC of 12-20 µg mL^{-1} based on paper disc diffusion tests (Lee et al., 2009). The *n*-hexane fraction of



(a) Whole body of a control nematode, showing normal body surface and annuli

(b) Whole body of a nematode after 24 h incubation in chloroform extract, showing annuli disappearance

(c) Section enlargement of a control nematode. Ring groove is evident

(d) Section enlargement of a nematode treated with chloroform extract. Body surface is smooth

(e) Further section enlargement of a control nematode, showing natural body surface texture

(f) Further section enlargement of a nematode treated with chloroform extract, showing the granular body surface

Figure 5. 5: Scanning electron microscope of *M. incognita*.

Image credits: Liu et al. 2013

D. crassirhizoma MeOH extract showed MIC of 15.6 µg/µL against *S. aureus* determined by

broth dilution method (Kwon et al., 2007). The plant extract showed bactericidal and bacteriostatic activity against *Streptococcus mutants* with MIC and MBC of 62.5 and 250 μg/mL, respectively) (Ban, Kim, Pandit, & Jeon, 2012). The MIC of *D. crassirhizoma* crude extract and hexane fraction was 0.008 mg/mL and 0.001 mg/mL, respectively against *Propionibacterium acnes* (Hisada & Noro, 1961)

Development of the chemometric approach to bioactive detection:

The standard purification approach is also a major challenge in the identification of the active components in TCM. TCM often acts in a combination of different components which are likely to be separated by extensive fractionation and sub-fractionation and therefore would not be detected in any bioassay-based identification strategy. In contrast, metabolomics simultaneously quantifies multiple small molecule types, such as amino acids, fatty acids, carbohydrates, or other products of cellular metabolic functions in a complex biological sample. Metabolite levels and relative ratios reflect metabolic function, and out of normal range perturbations are often indicative of disease. The progress in metabolomics has been largely driven by the development of mass spectrometry, and contemporary methods such as nuclear magnetic resonance (NMR), as well as various types of chromatography, spectrometry, and electrophoresis (Nagana Gowda and Djukovic, 2014). Combined analytical tools include gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled to mass spectrometry (LC-MS), capillary electrophoresis coupled to mass spectrometry (CE-DM), ultra-high performance liquid chromatography coupled to mass spectrometry (UPLC-MS), high-performance liquid chromatography-electrospray ionization coupled to mass spectrometry (HPLC-ESI-MS), high-performance liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry (HPLC/DAD/ESI-MS), and others.

One method of identifying compounds in complex mixtures is based on the application of metabolomic approaches. This is a high-throughput analytical technique that offers a global analysis of the products of all cellular metabolic reactions, regardless of the reactions that lead to their production (Perić-Concha & Long, 2003). To more accurately detect the lower concentration components of an extract, chemometric techniques can be used to profile every chemical in a mixture using chromatographic analysis coupled with spectrometric/spectroscopic techniques, so that fewer rounds of purification of the original extract is needed. The large data created from metabolomics can then be analysed by techniques such as principal component analysis (PCA) and the data created can be integrated with chemical data sets to predict the active components of the complex mixture without all the steps of bioassay-guided fractionation. This technique is called biochemometrics (Kellogg, Paine, McCune, Oberlies, & Cech, 2019). This technique makes the preliminary screens for effective antimicrobials more efficient and cheaper making research into natural extracts a more viable option and opens up new possibilities in natural product research.

This chapter focuses on the isolation and identification of the bioactive responsible for anti-MRSA activity of *D. crassirhizoma* observed in Chapter 2. The initial extractions of *Dryopteris crassirhizoma* (DC) were done following a bioassay guided purification and the most active fraction was further extracted. The fractions were simultaneously prepared for FIE-HRMS and UHPLC-MS. The data obtained from FIE-HRMS was randomised and assessed using multi-variate statistical approaches. Metabolites present only in active fractions were identified and used to search the literature, based on these, tentative identifications of the bioactive compounds were possible.

5.2. METHODS:

Part I: Traditional methods for isolation of bioactives

Extraction and purification:

Dried and powdered rhizome (800 g) of *Dryopteris crassirhizoma* was extracted sequentially (See Chapter 3). Approximately 93 g of *n*-hexane extract was fractionated using silica gel column chromatography (CC) (4×50 cm, 150 g of SiO_2,). The column was run four times and eluted with *n*-hexane-EtOAc (1:0 to 0:1, at 10 % gradient, 500×4 mL of each eluent), and EtOAc-MeOH (1:0 to 1:2, 500 mL×3 of each eluent) to yield 16 fractions (designated H for "*n*-hexane" and A through to P; thus, HA through to HP). Fraction HB (59.11g) was again subjected to silica CC (4×50 cm, 150 g of SiO_2,) eluting with *n*-hexane-EtOAc (1:0 to 3:20, 1% gradient, 500 mL×4 of each eluent) and EtOAc-MeOH (1:0 to 1:2, 5% gradient, 500 mL×5 of each eluent) to yield 8 fractions (1 through 8; thus HB1-HB8). The HB5 fraction (10g) fractionated again using silica gel CC (4×50 cm, 150 g of SiO_2,) eluted with *n*-hexane-EtOAc (1:0 to 1:10, 1% gradient, 500 m×4 of each eluent); *n*-hexane-EtOAc (1:10 to 0:1, 10% gradient, 500 mL ×4 of each eluent) and EtOAc-MeOH (1:0 to 1:2, 10% gradient, 500 mL ×2 of each eluent). This resulted in 14 fractions, a through to n; thus HB5a-HB5n.

B5d underwent a series of purification processes using silica CC (1.2×34 cm, 15 g of SiO_2). Eluting with *n*-hexane-DCM (1:0 to 0:1, 10% gradient, 15 mL×4 of each eluent) and DCM-MeOH (1:0 to 1:2, 15 mL×4 of each eluent); Semi-preparative HPLC (*see 4.2.6*), 20mg was run through Sephadex LH20 CC (34 × 1.2 cm) was used to eluted with chloroform ($CHCl_3$) and MeOH (1:1) sample collected every hour (Ren et al. 2016). Fractions B5d and B5e showed similarity in components detected by UHPLC-MS, B5e was further purified using silica CC same as B5d.

As no pure compounds were obtained, B5d and B5e was combined (~1g) and further fractionated with silica CC (4×33 cm, 150 g of SiO_2). Eluting with n-hexane-DCM (1:0 to 0:1,

10% gradient, 500 mL×4 of each eluent) and DCM-MeOH (1:0 to 1:2, 500 mL×4 of each eluent) yielding four fractions HB5d/e1 - HB5d/e8.

HB5d/e4 was further fractionated 10 mg through Sephadex LH20 CC (34 × 1.2 cm) was used to eluted with chloroform ($CHCl_3$) and MeOH (1:1) sample collected every hour yielding 2 fractions HB5d/e4AS and HB5d/e4BS. As an alternative approach, 60 mg with silica CC (1.5×30 cm, 6 g of SiO_2) eluting with *n*-hexane-MeOH (1:0 to 1:1, 0.5% gradient, 20 mL×5 of each eluent) yielding 5 fractions HB5d/e4A- HB5d/e4E.

HB5d/e6 was fractionated using preparative-thin layer chromatography (prep-TLC plates, 0.5 mm×20 cm×20 cm coated with silica gel 60 with F254 indicator (Material Harvest Ltd)) at a concentration of 10 mg/mL yielding 4 fractions HB5d/e6_1 through to HB5d/e6_4. The rest was run through silica CC (1.5×30 cm, 5 g of SiO_2) eluting with *n*-hexane EtOAc (1:0 to 1:1, 0.5% gradient, 20 mL×5 of each eluent) and EtOAc-MeOH (1:0 to 1:1, 10% gradient, 500 mL ×2 of each eluent) yielding 4 fractions HB5d/e6A- HB5d/e6D.

Part II: Chemometric approach for bioactive identification:

Fractionation:

A further 1g of HL was further fractionated on a silica CC (1.5×50 cm, 50g of SiO_2), eluted with 100% of 100mL *n*-hexane and EtOAc respectively, followed by EtOAc-MeOH (1:0 to 3:2, 1% gradient 100 mL of each eluent), yielding 8 fractions (HL1 through HL8). All crude fractions, n-hexane fractions and HL were further run through FIE-HRMS to generate metabolite fingerprints (Chapter 2; See 2.2).

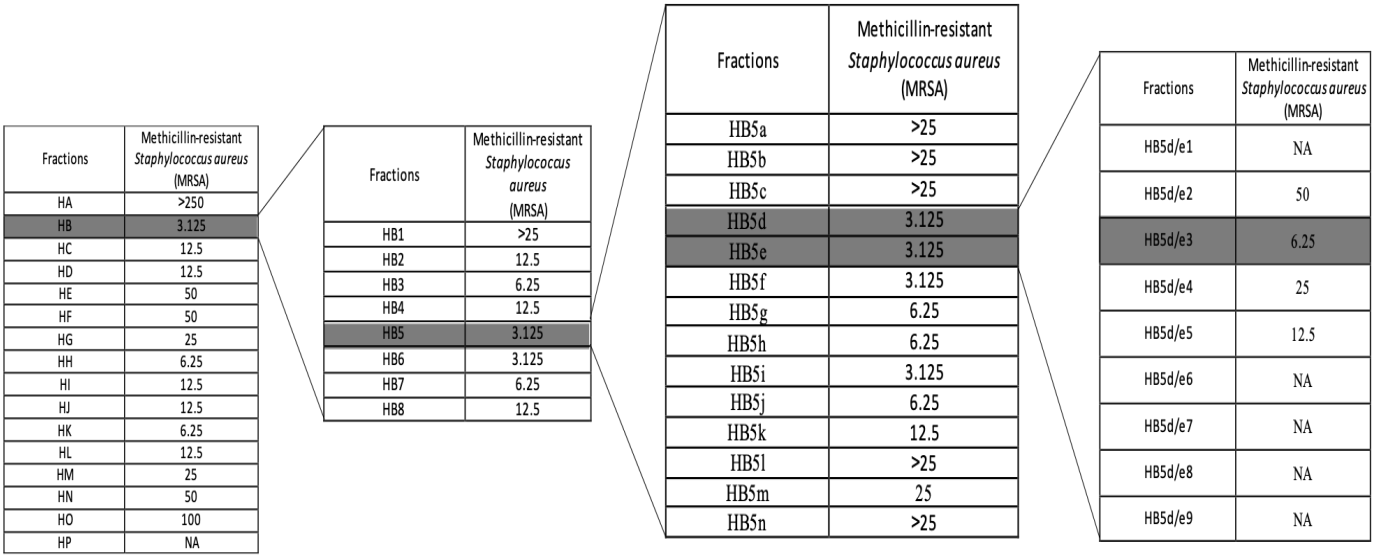
Data analysis:

Statistical tests were done using MetaboAnalyst 2.1. (Baptista et al., 2018) The data were then analysed using biochemometric analysis (Kellogg et al, 2016) to identify the active components.

5.3. RESULTS:

Part I: Traditional method for isolation of bioactives:

The crude extracts were screened for antimicrobial activities and the MIC for each was determined (See Chapter 3). Based on the MIC against MRSA (3.125 µg/mL) observed from the crude extracts we focused on the *n*-hexane extract for further purification. After a first round of purification of *n*-hexane extract all resulted fraction were assayed for MICs against SA and MRSA indicating that HB displayed the highest anti-microbial activity (Figure 5.7) HB was further purified, and the following anti-microbial screening suggested that fraction HB5 and HB6 had the highest activity against MRSA (Figure 5.7). The biochemistry of HB5 and HB6 the extracts were assessed using UHPLC-MS (Figure 5.8). HB5 was used for further purification based on its larger quantity and the similarity of its mass ions with HB6. Then, based on the MIC values, fractions HB5d and HB5e were chosen to be further purified. However, the biochemical components of the fractions as detected by UHPLC-MS showed that they were very similar (Figure 5.9) and so were combined and further fractionated. All fractions were tested for antimicrobial activity.



Fractions	Methicillin-resistant *Staphylococcus aureus* (MRSA)
HA	>250
HB	3.125
HC	12.5
HD	12.5
HE	50
HF	50
HG	25
HH	6.25
HI	12.5
HJ	12.5
HK	6.25
HL	12.5
HM	25
HN	50
HO	100
HP	NA

Fractions	Methicillin-resistant *Staphylococcus aureus* (MRSA)
HB1	>25
HB2	12.5
HB3	6.25
HB4	12.5
HB5	3.125
HB6	3.125
HB7	6.25
HB8	12.5

Fractions	Methicillin-resistant *Staphylococcus aureus* (MRSA)
HB5a	>25
HB5b	>25
HB5c	>25
HB5d	3.125
HB5e	3.125
HB5f	3.125
HB5g	6.25
HB5h	6.25
HB5i	3.125
HB5j	6.25
HB5k	12.5
HB5l	>25
HB5m	25
HB5n	>25

Fractions	Methicillin-resistant *Staphylococcus aureus* (MRSA)
HB5d/e1	NA
HB5d/e2	50
HB5d/e3	6.25
HB5d/e4	25
HB5d/e5	12.5
HB5d/e6	NA
HB5d/e7	NA
HB5d/e8	NA
HB5d/e9	NA

Figure 5.6: Bioassay guided purified fractions of n-hexane extracts (MIC µg/mL).

Note: NA: Not active

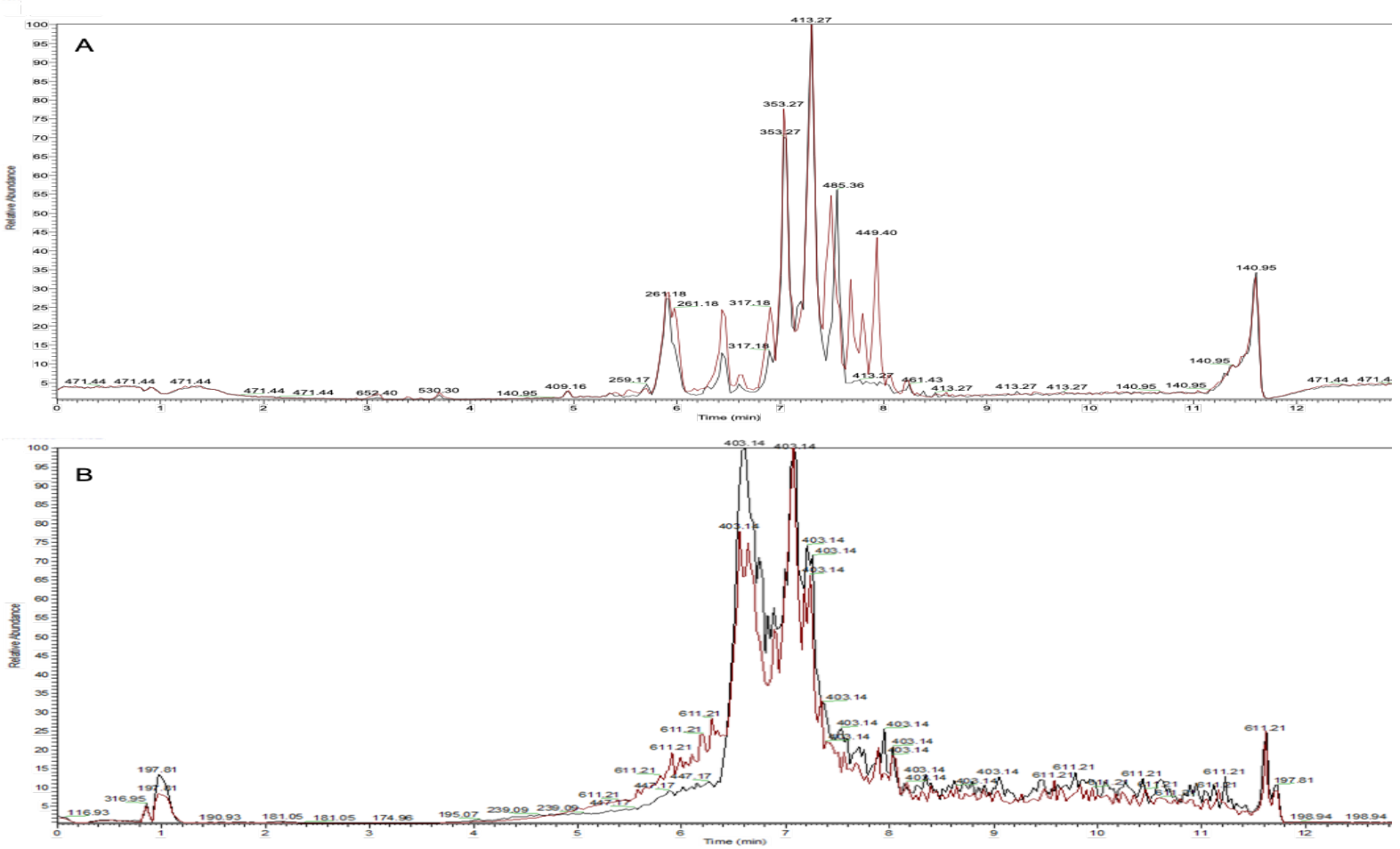


Figure 5.7: Total ion count (TIC) chromatograms of HB5 and HB3 obtained by UHPLC-MS.

A: Positive Ions of HB5 (black) and HB6 (red); B: Negative ions of HB5 (black) and HB6 (red)

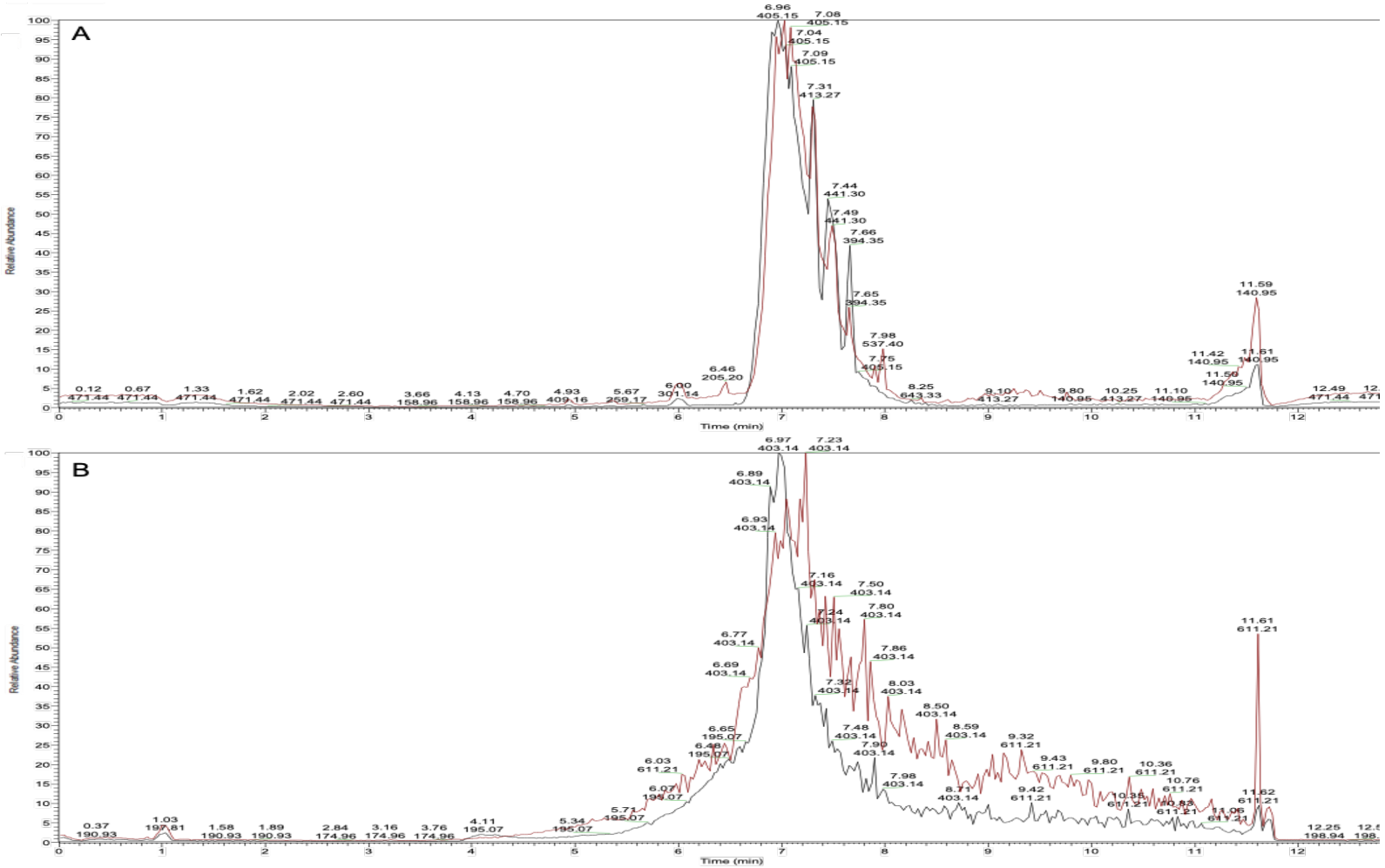


Figure 5.8: Total ion count (TIC) chromatograms of HB5d and HB5e obtained by UHPLC-MS.

A: Positive Ions of HB5d (black) and HB5e (red); B: Negative ions of HB5d (black) and HB5e (red)

All the fraction HB5d/e3-6 were further investigated via UHPLC-MS and it was observed that all these fractions had 2 common peaks with *m/z* 405.15417 and 419.16959 differing in their content. HB5d/e3 showed enrichment of *m/z* 405.15417, HB5d/e4 equal content of *m/z* 405.15417 and 419.16959, and HB5d/e5 and 6 showed enrichment of *m/z* 419.16959. These fractions underwent several alternative purification techniques as described in the methods section but failed to separate the compounds.

Finally, to identify the compounds HB5d/e3 were investigated via UHPLC-MS coupled with MS^2/MS^3 fragmentation (Figure 5. 10 and 5. 11). The structural information for characterization based on retention time, accurate mass, elemental composition, and multiple-stage mass data, (Table VII). This suggested that compounds were phloroglucinol derivatives, and when compared with the literature these were identified as norflavaspidic acid AB and flavaspidic acid AB (Ren, Quan, Wang, & Wang, 2016) (Figure 5.12).

Table VII: Identification of compounds using UHPLC-MS/MS

Compound	t_R, min	Observed mass	ESI-MS^n data (relative intensity %)
Norflavaspidic acid AB	6.73	405.15417	MS^2 [405]: 197 (100), MS^3 [405→197]: 179 (100), 155 (19), 151(8),113 (7)
Flavaspidic acid AB	6.96	419.16959	MS^2 [419]: 223 (9), 211 (61), 197 (100) MS^3 [419→197]: 179 (100), 155 (18), 151 (8), 113 (7)

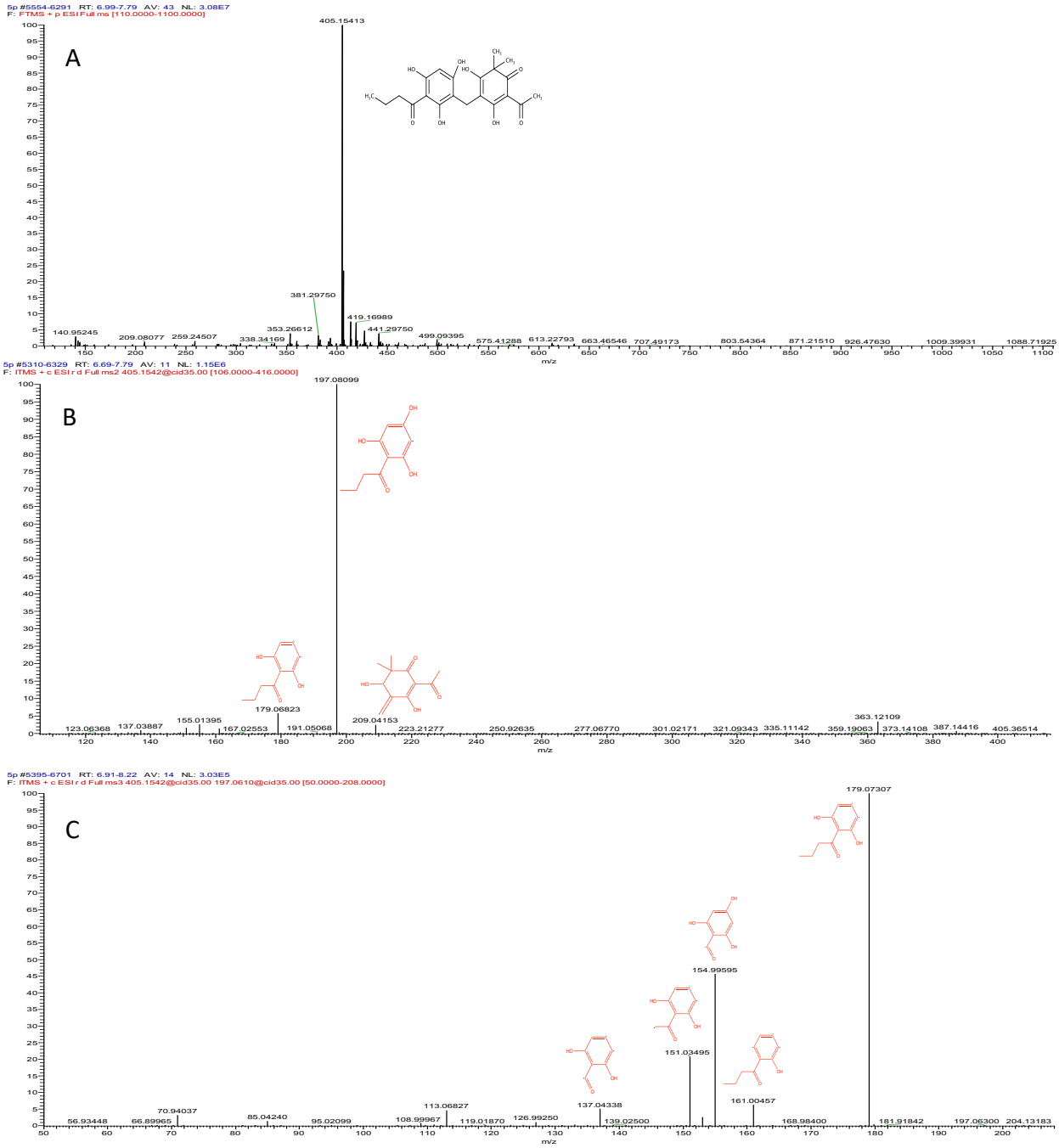


Figure 5. 9 : A: represents the conventional MS/MS spectra data of norflavaspidic acid AB ([MH]+obs. = 405.15413 amu).

A peak at *m/z* 419 and 441 were contaminants from the column. B: Represents the spectrum of the MS/MS/MS experiment in which norflavaspidic acid AB was fragmented by in-source fragmentation and its product ion peak at *m/z* at 405 was selected by Q1 and further fragmented in Q2. A second-generation product ion at *m/z* 197 was a result of breaking of the bond. C: represents the MS/MS/MS spectrum of the product ion peak at *m/z* 197. A main product ion at m/z 179 accompanied a neutral loss of 18 amu. The spectra also contain other peaks at *m/z* 154(loss of loss of 25 amu), *m/z* 151 (loss of 28 amu), *m/z* 161(loss of 18 amu) and *m/z* 137 (loss of 42 amu).

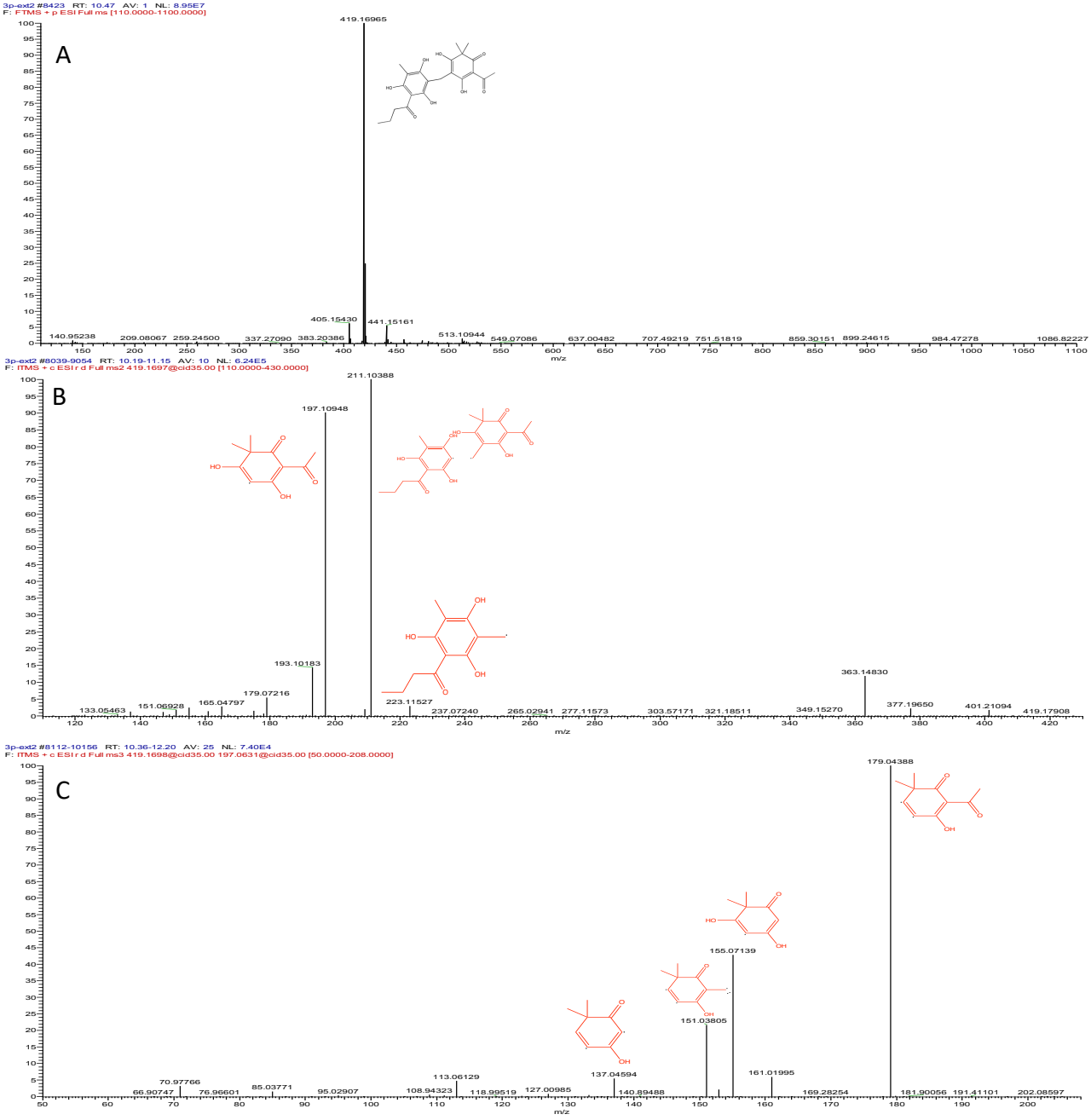


Figure 5. 10: A: represents the conventional MS/MS spectra data of flavaspidic acid AB ([MH]+obs. = 405.16965 amu).

A peak at *m/z* 441 was a contaminant from the column. B: Represents the spectrum of the MS/MS/MS experiment in which flavaspidic acid AB was fragmented by in-source fragmentation and its product ion peak at *m/z* at 405 was selected by Q1 and further fragmented in Q2. A second-generation product ion at *m/z* 211 and 197 was a result of breaking of the bond. C: represents the MS/MS/MS spectrum of the product ion peak at *m/z* 197. A main product ion at *m/z* 179 accompanied a neutral loss of 18 amu. The spectra also contain other peaks at *m/z* 155(loss of loss of 24 amu), *m/z* 151 (loss of 28 amu), *m/z* 161(loss of 18 amu) and *m/z* 137 (loss of 42 amu).

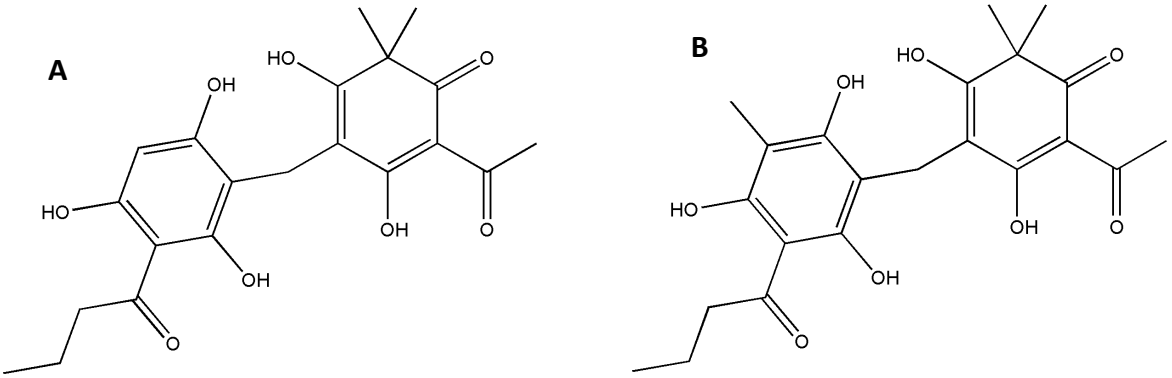


Figure 5.11: The chemical structures of the bioactive natural products isolated from *D. crassirhizoma*

A: Norflavaspidic acid AB; B: Flavaspidic acid

Part II: Chemometric approach for bioactive identification:

Column chromatography generated 8 fractions of HL: HL1 to HL8. Their antimicrobial activity was again tested with and the resulting MICs are recorded in Table VIII.

Table VIII: Antibacterial activity (MIC µg/mL) of HL fractions.

Note: ND: no detected activity in this concentration; Gentamycin sulphate:3.13 µg/mL

Fractions	*Staphylococcus aureus*	Methicillin-resistant *Staphylococcus aureus*
HL1	ND	ND
HL2	>25 µg/mL	25 µg/mL
HL3	>25 µg/mL	>25 µg/mL
HL4	>25 µg/mL	>25 µg/mL
HL5	25 µg/mL	25 µg/mL
HL6	25 µg/mL	25 µg/mL
HL7	12.5 µg/mL	12.5 µg/mL
HL8	25 µg/mL	12.5 µg/mL

Based on these MICs, metabolomic approaches were then used to target the possible compounds within the fraction that might be responsible for antimicrobial activity against MRSA.

Each fraction was profiled by FIE-MS and the resultant mass spectra were assessed using multi-variate statistical approaches. Principal component analysis (PCA) of the four fractions, predicting their relatedness (Figure 5.13). The *n*- hexane(H), DCM(D), ethyl acetate(E) and methanol(M) fractions showed clear separation on the PCA plot indicating that the variation in these sample metabolites were was significantly different from each other (Figure 5.13; A). Partial least square discriminant analysis (PLS - DA) of the *n* - hexane fractions (Figure 5.13; B) with significant activity showed that the HB was completely different from all the other fractions; especially, HA, fraction is known for its lack of active antimicrobial compounds. The proximity of the HH and HJ; HK and HL fractions indicates that they are groups of similar compounds and tellingly, all had the same MICs (Figure 5.13)

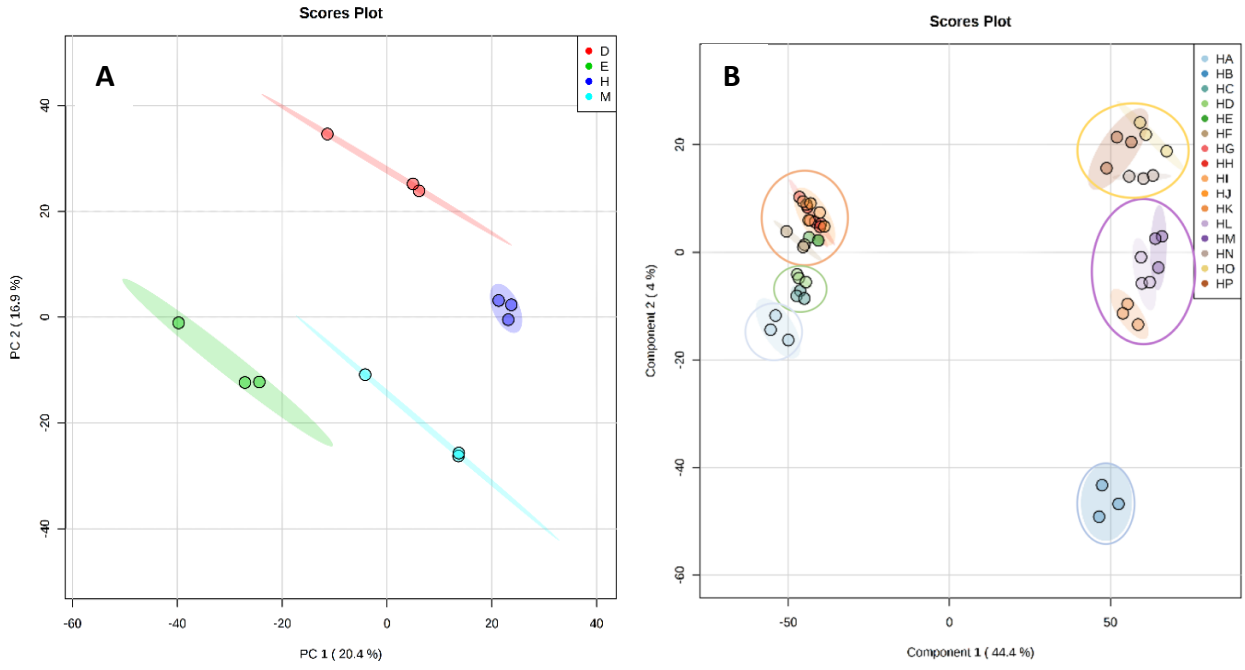


Figure 5.12: Multi-variate statistical approaches for different fractions of *D.crassirhizoma.*
(A) Principal component analysis (PCA) of the different crude fractions score plots (n = 6 and 95% confidence interval illustrated, clear outliers removed) of normalized m/z intensities of metabolites extracted from the fractions. (B) Partial least squares discriminant analysis (PLS-DA) of the *n*-hexane fractions score plot based on the top two components. Plots indicate metabolome differences between the different fractions

Next, metabolite profiles for the HL fractions were obtained and assessed by multivariate approaches. The PCA score plot though shows close proximity of HL5, HL6,HL7 and HL8 forming a group while HL3 and HL4 forms another group and HL2 is completely by itself (Figure 5.14). The major sources of variation were between active HL and less-active HL fractions were defined. These were compared across the fractions using a heat-map. HL7 and HL8 as they had the best MIC in comparison to the rest of the fractions and sources of variation for these fractions were distinct from other fractions (Figure 5.15). The masses picked for further investigation were significantly abundant in HL7 and HL8 fractions, whilst absent in other fractions, based on the heatmap analysis we targeted predict that *m/z* 625.228 and 627.235 as possibly could be responsible for the bioactivity.

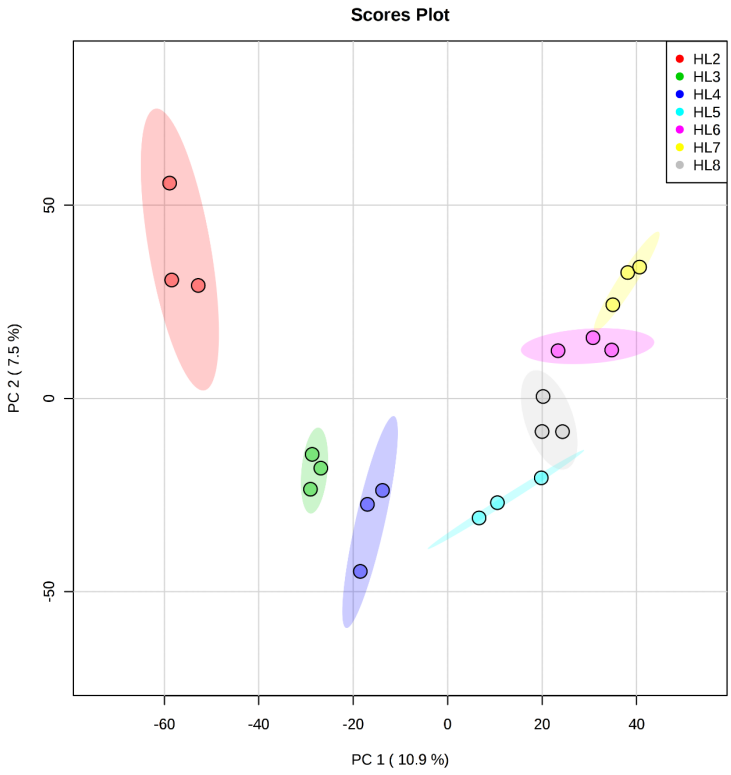


Figure 5. 13: Multi-variate statistical approaches for HL fractions of *D.crassirhizoma*.

Principal component analysis (PCA) of the different HL fractions score plots (n = 6 and 95% confidence interval illustrated, clear outliers removed) of normalized m/z intensities of metabolites extracted from the fractions. Plot suggests the metabolomic differences in different fractions

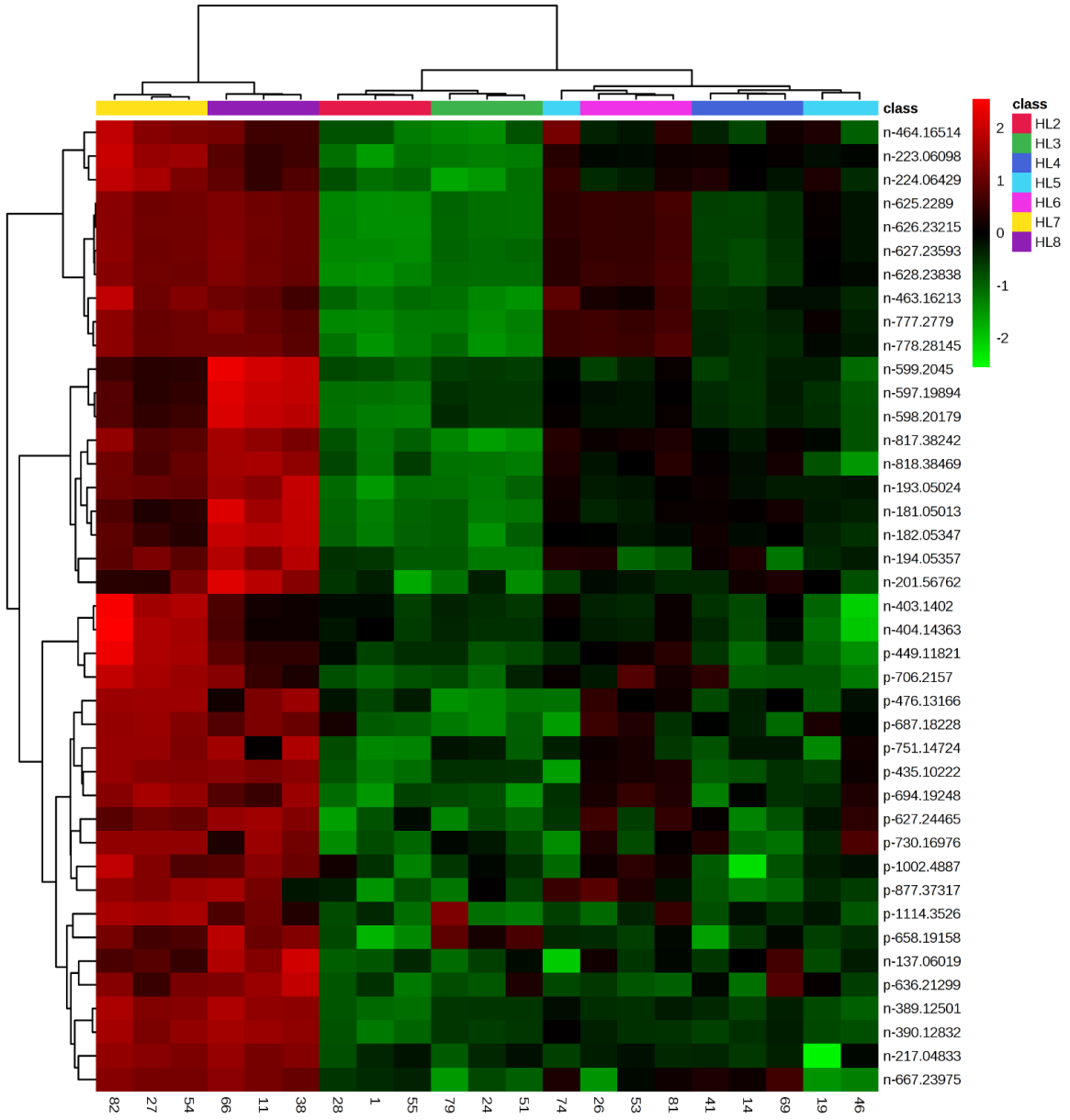


Figure 5. 14: Heat map showing cluster analysis of top 50 significant masses of HL7 and HL8 in comparision to other fractions.

The selected fractions were further analyzed under UHPLC-MS individually to identify the abundance of the observed *m/z* and underlying other masses present in the fractions. The most abundant peak in the HL7 extract was *m/z* 419.169 followed by *m/z* 857.299 and 649.225 (Figure 5.16). While in extract HL8 the most abundant peak was *m/z* 649.225 followed by *m/z* 419.169 and 857.299 (Figure 5.17). This suggests that masses other than *m/z* 649.225 present in the fraction could also contribute to the bioactivity.

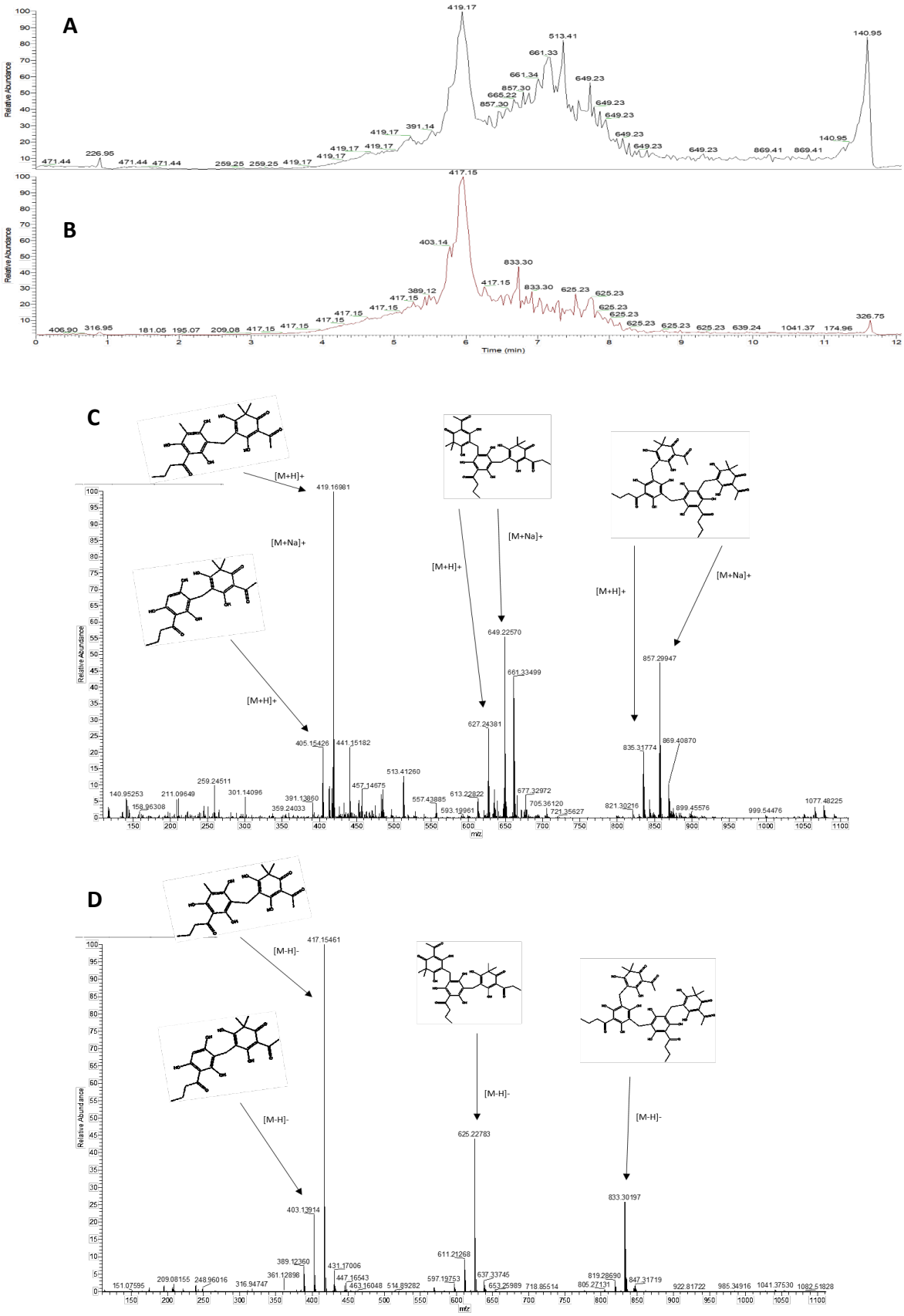


Figure 5. 15: UHPLC-MS analysis of HL7.

A: Total positive ion count of HL7, B: Total negative ion count of HL7, C: Mass spectrum of positive ion mode of HL7, D: mass spectrum of negative mode of HL7

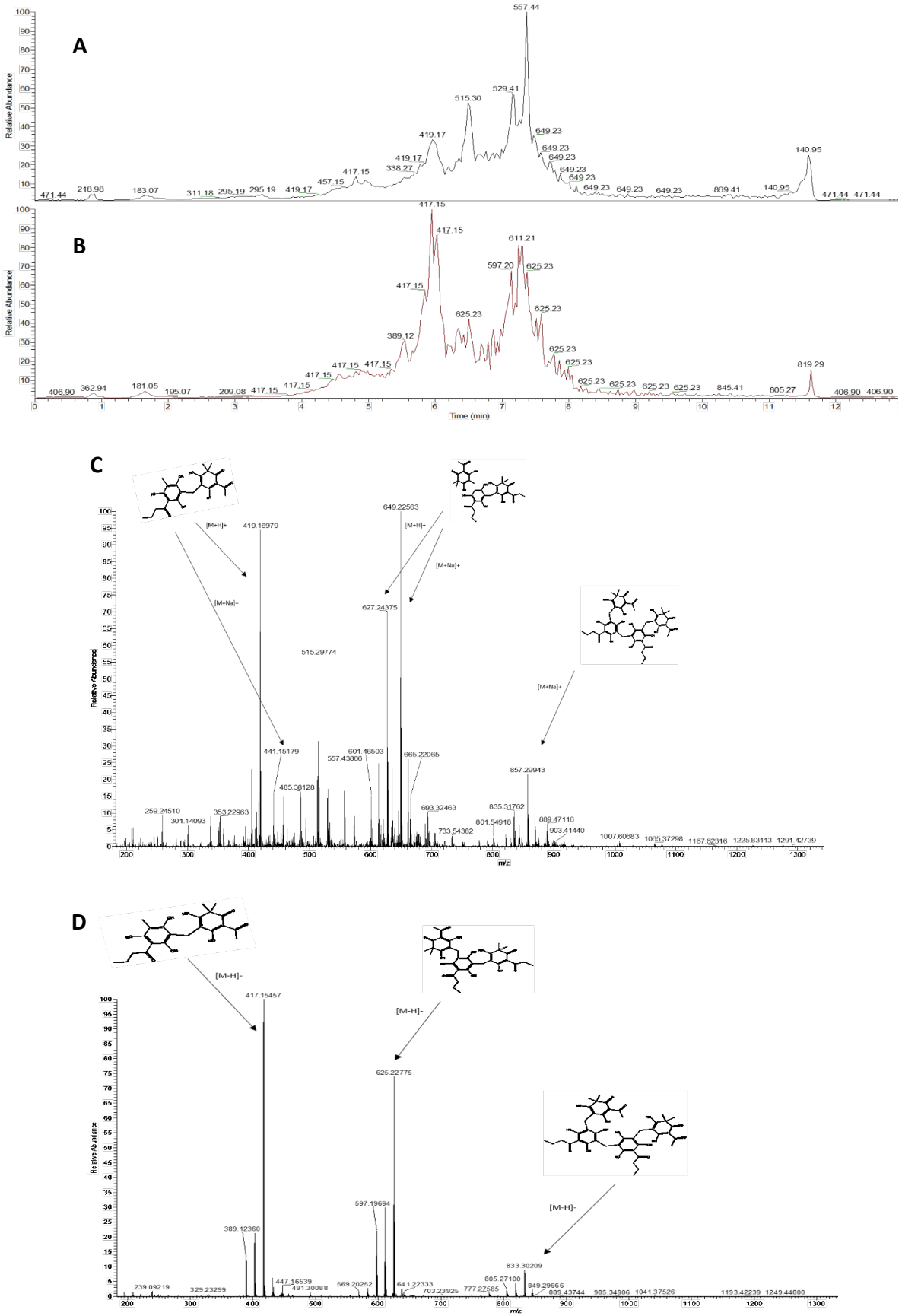


Figure 5. 16: UHPLC-MS analysis of HL8.

A: Total positive ion count of HL8, B: Total negative ion count of HL8, C: Mass spectrum of positive ion mode of HL8, D: mass spectrum of negative mode of HL8

On exploring previous literature and different metabolite databases we predicted that the structures of the compounds present in the extracts based on parental ion masses. Compounds detected based on ion spectrum of UHPLC-MS could be Filixic acid ABP *(m/z* 649.225), Flavaspidic acid AB (*m/z* 419.169), and Dryocrassin ABBA (*m/z* 857.299) (Na et al., 2006; Richter, Raschdorf, v. Euw, Reichstein, & Widén, 1987; Juan Wang et al., 2017) (Figure 5.18)

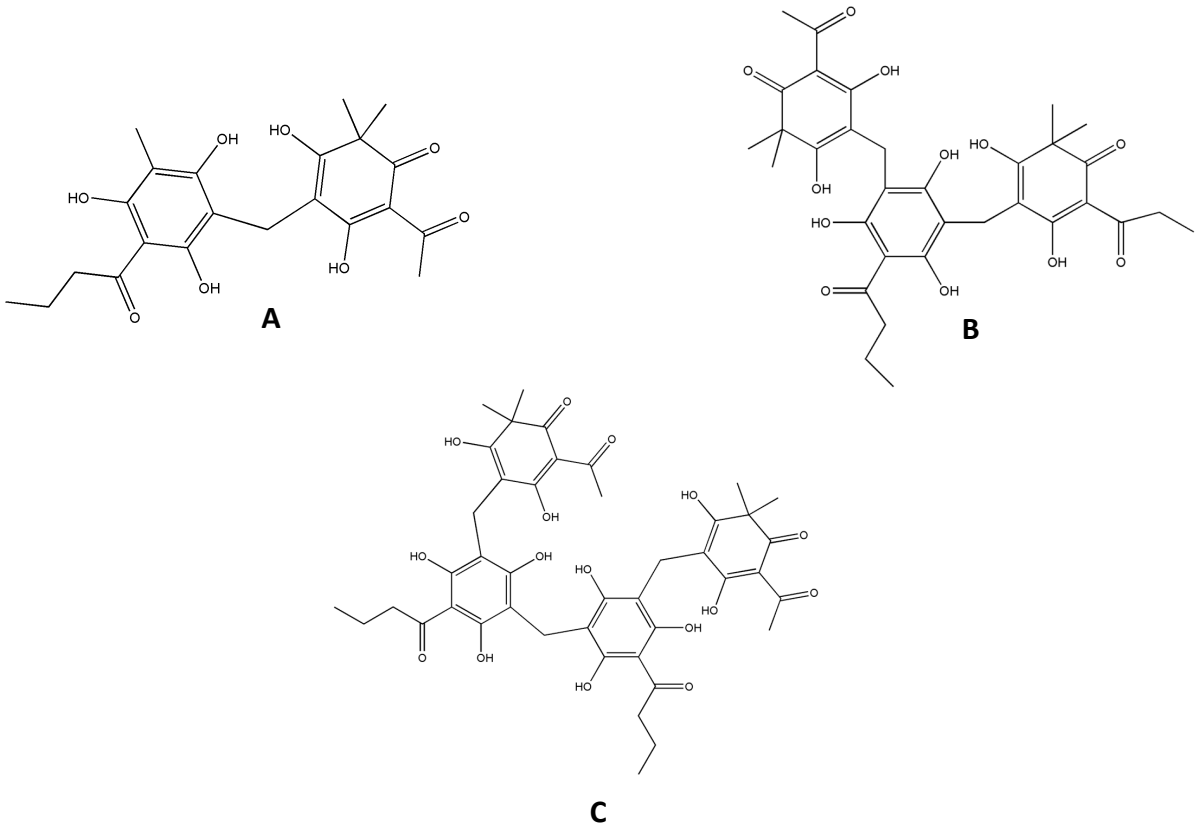


Figure 5. 17: The chemical structures of identified compounds.

A: Flavaspidic acid AB, B: Filixic acid ABP, C: Dryocrassin ABBA

5.4. DISCUSSION

The antimicrobial screening of *n*-hexane and DCM extracts of *D. crassirhizoma* showed activity against MRSA (USA300). Due to its low MIC value, the *n*-hexane extract was further sequentially fractionated, guided by bioassays, to ultimately yield a pair of fractions HB5d/e3 and HB5d/e5 with the best activities (MIC 6.25 and 12.5 µg/mL respectively).

After several failed attempts at purification, the HB5d/e3 fraction was identified using UHPLC-MS and MS^2/MS^3 and compared with the literature which indicated them to be phloroglucinols namely norflavaspidic acid AB and flavaspidic acid AB (Figure 5.12).

Norflavaspidic acid AB is an example of acyl phloroglucinol dimers formed by a methylene linkage. It has been reported to have inhibitory effects on melanin production by melanoma B16F10 cells with IC_{50} values of 181.3 µM (Pham, Kim, Lee, Min, & Kim, 2017). It also shows inhibitory effects against human leukemia Reh cells with IC_{50} of 32.2µg/mL (Ren et al., 2016). It has been reported to have inhibitory activity against the pro-apoptotic factor - FAS - of IC_{50} 29.7µM (Na et al., 2006).

Flavaspidic acid AB is an acyl phloroglucinol dimers, similar to norflavaspidic acid but with an added methylene group. It has been reported to exhibited a potent antioxidant activity against the LPO inhibitory test with IC50 values of 13.1 mM (Lee et al., 2003). It can induce IFN-α, IFN-β, and IL1-β expression in porcine alveolar macrophages, which could contribute to inhibition of porcine reproductive and respiratory syndrome virus (PRRSV) replication (Yang et al., 2013) It also shows inhibitory effects against human leukemia Reh cells with IC50 of 35.3µg/mL (Ren et al. 2016). Its activity against the pro-apoptotic ligand FAS had an IC_{50} of 28.7µM (Na et al., 2006). The effect of flavaspidic acids on cellular respiration and oxidative phosphorylation in isolated hepatocytes and even, at moderate concentrations, uncouples oxidative phosphorylation in isolated hepatocytes (Burnett, Lysenko, & Ockner, 1979). Flavaspidic acid AB is reported to have antibacterial activity based on when a paper disc

diffusion assays with MICs ranging between 12-20 µg/mL depending on the microorganism being tested (Lee et al., 2009).

A key feature of TCM is the use of blends of different herbs to make a complex mixture representing multiple interacting components. For example, *Fritillaria* bulbs are frequently found in many proprietary formulations for acute bronchitis and tuberculosis, such as San She Dan Chuan Bei Mu Ye (contains *Fritillaria ussuriensis* and *Zaccys dhymnades* bile juice), Si Shun Tang (include Zi Wan (Purple Aster Root), Jie Geng (Platycodon), and Gan Cao (Licorice Root)) and Chuan Bei Pi Pa Lu (*Eriobotrya japonica* leaf (Pi Pa Ye) *Typhonium flagelliforme* rhizome (Shui Ban Xia) *Fritillaria ussuriensis* bulb (Ping Bai Mu) *Platycodon grandiflorum* root (Jie Geng)). Their therapeutic effect is often associated with the combined action of several active ingredients, just as it can be caused by a single compound that the herb contains (Wang et al., 2017). Such complexity massively impedes the definition of the active components using the classical, bioactivity guided fractionation and purification strategy which is ideally suited to defining single active chemicals. Thus, we attempted an alternative approach which maintains the complexity of extracted but uses metabolomic and chemometric methods to define active component. The expansion of the 'omics technologies has allowed metabolomic approaches to be exploited in the analytical field. Thus, metabolomics can be used to explore the metabolite changes occurring in biological systems using chromatography techniques (LC-MS; GC-MS), nuclear magnetic resonance (NMR) and mass spectrometry (Fiehn, 2002). Metabolomics is used in medicine, microbiology and genomics. In pharmacology, it is utilised for drug toxicity research, diagnoses, and drug discovery (Lindon et al., 2000; Xia et al., 2009). The second-best group of H - fractions (MIC: 6.25 µg/mL) contained HH, and HK. PLS-DA, indicated that HH, HG and HI formed one cluster while HK, HL and HM formed another. The HL fraction was chosen to be the one to be subjected to further fractionation due to the amount

of material in the extract and as it was extracted with different polarity of solvents to HB, would be unlikely to contain similar active metabolites.

The fractionation followed by antimicrobial assay performed on HL yielded the MICs that are presented in Table VIII. It was separated into 8 fractions (HL1 to HL8) by column chromatography, with varying MICs (from 12.5 µg/mL to >25 µg/mL) against the two bacterial strains. The differences in the MICs between the HL fraction (6.25 µg/mL) and the following separated HL2 to HL8 might be due to acting in synergy against MRSA in HL and this interaction is lost with fractionation. This highlights the need to develop a chemometric approach to bioactive discovery as attempted in this chapter.

In this study, data acquired from FIE-HRMS of HL fractions of *D. crassirhizoma* were first assessed by PCA. The data from *D. crassirhizoma* indicated that the clusters formed from *n* - hexane, DCM, EtOAc and methanol fractions with each being significantly different from the other (95% CI) (Figure 5.13).

Another chemometric analysis was performed on the data from *n* - hexane fractions of *D. crassirhizoma*. PLS - DA uses many linear regression models to find the largest covariance of the data set (X) and the class (Y) (Xia et al., 2009). The variables of the data set are grouped into averages called scores. The analysis therefore provides a "supervised" view of the data where class differences are highlighted. The 2D biplot showed the HA - HP fraction grouping (Figure 5.13) shows that the HB activity is the highest, confirming the data from the MICs. HL and HK clusters are close to that of HB; where the active compounds have been defined. It may be assumed that due to the proximity of the HL, HK and HB fractions, they share common antimicrobial compounds. PLS - DA of HL fractions showed that these were distinctive (Figure 5.13).

Further investigation of HL involved further fractionation based on polarity with subsequent estimations of MICs for each fraction. All the fraction was run through FIE-HRMS and the

major sources of variation assessed (Figure 5.15). Later the most active fractions HL7 and HL 8 was run through UHPLC-MS to further select the abundant mass ions. Based on the data obtained and searching the databases and previous literature, 3 compounds were identified.

Flixic acid ABP is the second most abundant compound in HL7 and HL8 (Figure 5.16 and Figure 5.17), in the UHPLC-MS spectra. Flixic acid ABP is known for its antimicrobial properties and has been identified in *D. crassirhizoma.* Its MIC against *S. aureus* strains was reported to be 2.5 µg/mL (Qi et al., 2017)

Flavaspidic Acid AB, the most abundant compound according to UHPLC-MS, is also known for its antimicrobial and antioxidant activities (Figure 5.16 and Figure 5.17). It is a metabolite that can be found in the rhizomes of *D. crassirhizoma* and inhibits the replication of respiratory syndrome virus (PRRSV) (Yang et al., 2013). It was first defined in an ethyl acetate - soluble fraction of the rhizomes of *D. crassirhizoma* based on its property as a potent antioxidant based on a Lipid Peroxidation (LPO) inhibition assay with IC50 values of 12.9 µm, (Lee et al., 2003). This activity may be due to the iron chelating activity of phenolic compounds which will suppress the ability to generate free radicals (Van Acker, 1998). Considering its anti-microbial properties, disk diffusion tests against two strains of *S. aureus: Staphylococcus aureus* KCTC 1916 and *Staphylococcus aureus* KCTC 1928 mutants suggested inhibition zones of 16 and 19 mm respectively. As *Staphylococcus aureus* KCTC 1928 mutant is a MRSA bacterium this shows the clinical potential of flavaspidic acid AB (Lee et al., 2009). Given these observations, flavaspidic acid AB is likely to contribute to the anti- *S. aureus* and MRSA activities in the HL fraction of the *n* - hexane fraction.

Dryocrassin ABBA, is third targeted compound (Figure 5.16 and Figure 5.17), This has already shown an antimicrobial activity for bird flu virus and the downregulation of cytokines IFN - γ and TNF - α, which are associated with the development of influenza virus (Hagau et al., 2010). A possible connection of dryocrassin and the suppression of *S. aureus* and MRSA infections

has been suggested in a recent study where dryocrassin ABBA has a strong inhibitory action on Von Willebrand factor-binding protein (vWbp), a protein secreted by *S. aureus* and has been considered a factor for the decrease of emerging resistance strains such as MRSA (Li et al., 2019). However, such activities would be not have been revealed by the screens used in the experiments used in this chapter but highlight the potential of TCM based on *D. crassirhizoma.* MS based techniques to provide a rapid investigation to any new natural product identification, as it is more sensitive compared to other analytical methods. High resolution MS (3-5 ppm resolution) as used allows the identity of the metabolites to be suggested but depends on the metabolite being already lodged in databases. Therefore, high-resolution MS suffers from not providing an unambiguous identification of *m/z* and a failure to report novel compounds. For unknown compounds, not contained in the libraries, their structure assignment by MS is still considered a challenge(Wang et al., 2017). As a result, Currently, MS/MS fragmentation is routinely applied in natural product identification and characterization, in which MS/MS data are definitive characteristics of a molecule leading to dereplication in combination with library searches.

5.5. CONCLUSION

The increasing interest the world is showing into TCM is due to its undiscovered potential as a multi-target therapeutic. *D. crassirhizoma* has been demonstrated to contain multiple antimicrobial components that either in association with each other, or separately, can successfully target MRSA and be potentially turned into a novel drug. Based on the MIC of *D crassirhizoma* against MRSA obtained in Chapter 3, the objective of this chapter was to characterize the bioactive responsible for the activity. Part I of the the chapter demonstrated the novel bioactive fraction of *D. crassirhizoma*. The fraction showed MIC 6.25 µg/mL was further characterized using UHPLC-MS coupled with MS^2/MS^3. Norflavaspidic acid AB and Flavaspidic acid AB was identified as the major components of the fractions. There were several alternative methods used to separate the fraction but failed. Therefore, we tentatively conclude the two compounds identified are responsible for the anti-MRSA activity.

The mode of action of norflavaspidic acid AB and flavaspidic acid AB need to be investigated and in the next chapter, this will be explored using metabolomics.

Part II of the chapter, chemometric assessments of metabolomic data been demonstrated as a potential method to identify bioactives in a chemically complex fraction. The success of this approach was aided by the existence of a comprehensive literature which allowed the key sources of variation to be identified and linked to known anti-microbial activities. Clearly, this prevents the rapid definition of novel compounds. However, this approach could be extended to unknowns. Thus, some novel compounds could be present in active fractions and only indicated by their *m/z*. These would be still targeted for identification by tandem MS or NMR. They could still be purified, and their effects assesses using standard synergistic assays with bioactives from the extract or TCM mix. Finally, the chemometric process could be further improved to make it much more efficient. For example, machine learning approaches could be

used to identify variables present only in active fractions which could be immediately be used to interrogate natural product databases.

6

Metabolomics:

Assessments of mode of action

Abstract

In response to the global need for new anti-microbial that can effectively combat the new strains of drug-resistant "superbugs", our study uses metabolomic approaches to exploit the potential of TCM. There are several strategies that can be used to define drug modes of actions. Traditional means include simple assessments of changes in cell phenotype upon drug treatment, drug bioactivity but more molecular approaches include affinity chromatography, radio-labelling, and cell-based affinity tagging. However, genomic and post-genomic approaches could provide wider assessments of many effects on a given drug lead on the target organism or cell (Baptista et al. 2018).

Extracts from *Dryopteris crassirhizoma* Nakai exhibited potent activity (MIC 3.125 μg/mL); bioassay-guided purification led to their chemical identification. High-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) indicated the key bioactive to be phloroglucinols. We here use metabolomic approaches based on flow infusion ionisation electrospray high-resolution mass spectrometry (FIE-HRMS). to suggest possible mode(s) of action for the bioactive fractions HB5d/e3(A3) and HB5d/e5 (A5) fraction of *Dryopteris crassirhizoma*. The major sources of variation and biochemical pathway assessments, anti-MRSA activity was linked to the altered amino acid processing. Genomic assessments of resistant mutants will be used to further confirm the predicted target.

6.1. INTRODUCTION

A vitally important aspect of research leading to new drug leads or the repurposing of existing drugs is to fully comprehend, at least as far as possible, their mechanisms of action. This can prevent problems with toxicity at the early stages of clinical trials. For example, the drug target must not show such similarity with human homologues so that is could compromise the drug safety. There some key features that the drug targets should display to improve the likelihood of a drug-lead being taken forward for development as an anti-microbial. Kana et al. defines the "ideal" target as a macromolecule that 1) acts in the growth, survival and latency of the bacteria, 2) regulates crucial checkpoints of bacterial metabolism, 3) has a low tolerance for mutation and 4) is found at targetable locations within the cell. Given the importance of defining the mode of action and likely targets for new drug leads, we here consider the known modes of action for existing MRSA targeting drugs prior to moving to assess likely mode(s) of action for the A3 fraction of *Dryopteris crassirhizoma* (See 5.1).

Different means of mode of action studies

There are several strategies to define mode of actions. Traditional means include simple assessments of changes in cell phenotype upon drug treatment, drug bioactivity but more molecular approaches include affinity chromatography, radiolabeling, and cell-based affinity tagging (Azad & Wright, 2012). However, it is increasingly being understood and genomic and post-genomic approaches could provide wider assessments of many effects on a given drug lead on the target organism or cell

Omics technologies have revolutionized many fields of biology and are expected to play a key role in the effective control of pathogens through the development of highly specific antimicrobial agents (Figure 6.1). High-throughput molecular techniques, colloquially called "omics" methods, have greatly increased our ability to characterize the taxonomic and genetic

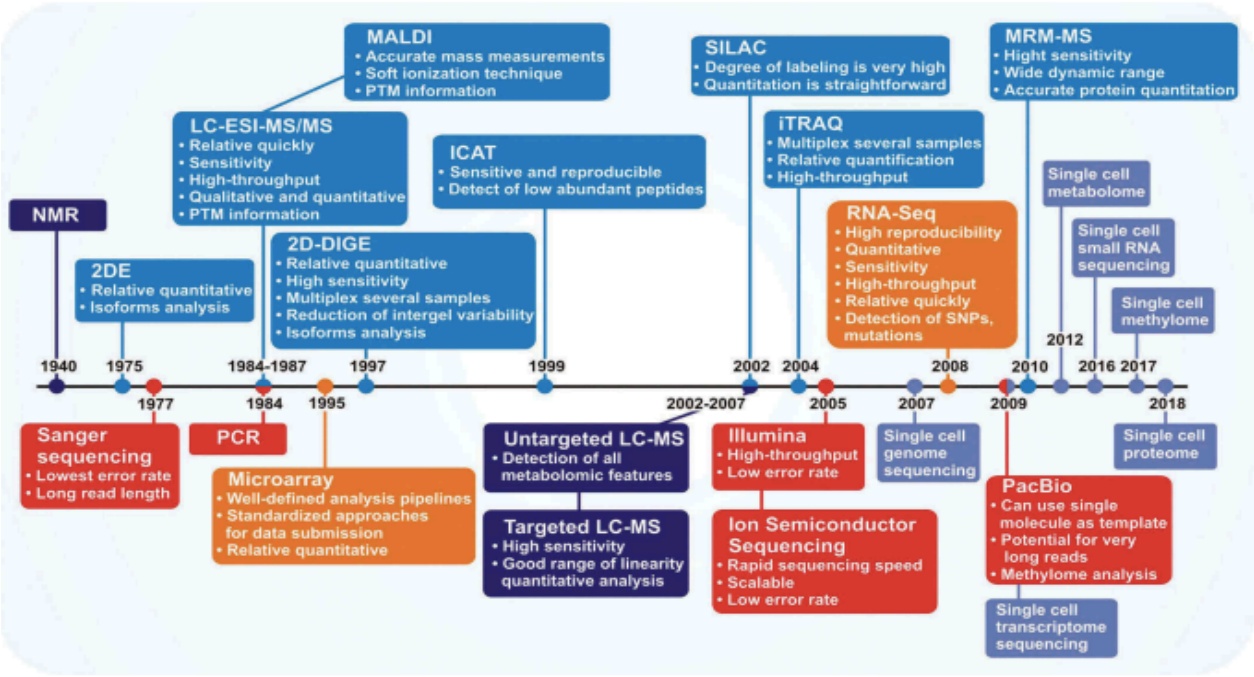


Figure 6.1: Timeline of the different omics methods.

The milestones are shown in different colours for the works in genomics, transcriptomics, proteomics, metabolomics, and single cells (Chernov, Chernova, Mouzykantov, Lopukhov, & Aminov, 2019)

structure of bacterial communities, to estimate their functional capabilities and to evaluate their responses to stressors or pathogens (Tsouh Fokou et al., 2015). Some of the omics methods developed to date are gene amplicon sequencing, shotgun metagenomics, transcriptomics, proteomics, and metabolomics.

Genomics: The tremendous progress in genome analysis in the past decade has had a major impact on all biological sciences, including drug characterization (Figure 6.2). Genomic information is important in the identification of promising candidate targets for antimicrobials. Identification of genes essential for the survival of pathogens is clearly important for the development of screening platforms to discover antimicrobial compounds targeting the corresponding products encoded by these genes (Brown & Wright, 2016; Fields, Lee, & McConnell, 2017). Several pharmaceutical companies have started sequencing programs and have adapted their antibacterial drug discovery process to maximize the use of this unprecedented information. With the ever-increasing number of multidrug-resistant bacterial

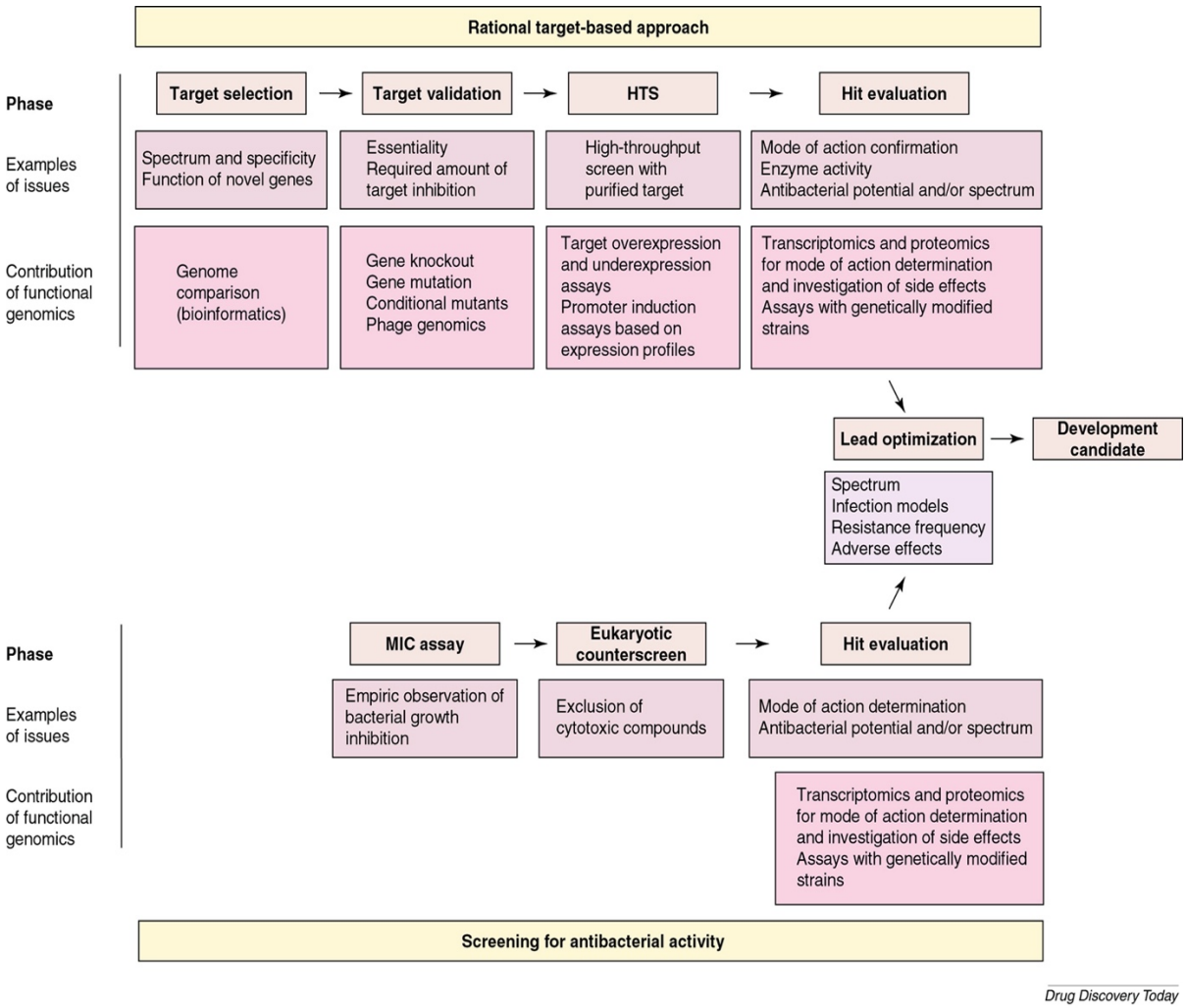


Figure 6.2: Various functional genomics techniques implemented in the modern antibacterial drug discovery process.

isolates, the pharmaceutical industry has embraced genomic information as the basis for a rational, target-directed antibacterial drug discovery strategy to complement the classic empirical approach (Freiberg & Brötz-Oesterhelt, 2005; Livermore, 2003; Spellberg, Powers, Brass, Miller, & Edwards, 2004). Comparative genomic analysis of strains with different sensitivities to antimicrobials is of importance for the identification of complex antimicrobial resistance mechanisms. Other components of microbial communities are the virome and vesicles which affects the population density of cellular organisms and, via transduction, provides horizontal gene transfer (HGT), including transfer of genes encoding antimicrobial resistance (Haaber et al., 2016; Kim et al., 2018). Genomics studies have also made a significant contribution to the understanding of the role played by membrane vesicles in antimicrobial resistance.

The efforts undertaken by GlaxoSmithKline (GSK) between 1995 and 2001 offers a good example of the use of comparative genomics in identifying targets for the development of broad-spectrum antimicrobials. Over this period, researchers at GSK mined the genomes of *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumonia*, *Staphylococcus aureus* and *Enterococcus faecalis* to identify highly conserved genes. In total, more than 300 genes were identified as potential targets, resulting in 67 high throughput screens. These screens led to the identification of sixteen hits that gave a positive result *in vitro*, five of which were developed as lead compounds. However, none of these compounds advanced into clinical testing. GSK subsequently reorganized its antibiotic discovery program to employ more traditional methods based on using chemical synthesis to develop novel agents that inhibit known targets using novel mechanisms (Payne, Gwynn, Holmes, & Pompliano, 2007).

Transcriptomics: is the analyses of a complete set of transcripts produced by the cell under defined environmental conditions. Transcripts are assessed as a genome-wide level, both qualitatively (which transcripts are present, identification of novel splice sites, RNA editing

sites) and quantitatively (how much of each transcript is expressed). Analysis of gene expression in response to antimicrobials in conjunction with bioinformatics resources may help to reveal the mechanisms of antimicrobial action. Microchip technology based on hybridization of cDNA with oligonucleotide probes can be used for large-scale gene expression studies (Batchelor et al., 2008; Bruant et al., 2006; Freiberg & Brunner, 2002). Gmuender and others revealed differences in the cellular response of *Haemophilus influenzae* to DNA gyrase inhibitors (novobiocin and ciprofloxacin) using DNA microarrays (Affymetrix chips) and 2D-PAGE (Gmuender et al., 2001). This study was one of the earliest works suggesting that the microbial response to antimicrobials and mechanisms of antimicrobial resistance are not simply limited by the boundary's antimicrobial-target interaction and could be more complex, involving a multitude of other genes.

The use of hybridization-based techniques in transcriptome analysis is gradually being replaced by global RNA sequencing (Duggan, Bittner, Chen, Meitzer, & Trent, 1999; Ozsolak & Milos, 2011; Schulze & Downward, 2001; Wang, Gerstein, & Snyder, 2009). The RNA-seq method provides a deep quantitative analysis of gene expression and allows the profile of non-coding RNAs involved in the regulation of gene expression to be evaluated. Small RNAs (sRNAs) in the extracellular vesicles of microorganisms are of particular interest because they are considered to be mediators of cell reprogramming in response to changes in environmental variables (Choi, Kim, Hong, & Lee, 2017; Dersch, Khan, Mühlen, & Görke, 2017). The involvement of vesicular sRNAs in response to antimicrobial agents and the corresponding development of resistance has recently been demonstrated for *Acholeplasma laidlawii*, where the wild-type and resistant strains were grown under antibiotic-free and antimicrobial treatment (ciprofloxacin, tetracycline, and melittin) conditions. Some differentially expressed sRNAs were associated with genes conferring antimicrobial resistance, including metal-dependent β-lactamases, MATE-family proteins, and ABC-transporters (Chernov et al., 2018). Validation

of transcriptome analysis can be provided by RT-PCR and using mutagenesis techniques such as Tn-seq, INseq, TraDIS, or HITS (Moule et al., 2014; Rajagopal et al., 2016; Slavokhotova, Shelenkov, & Odintsova, 2015; Van Opijnen & Camilli, 2013).

Proteomics aims to quantify large numbers of peptide and protein abundance, modification, and interactions in cells, tissues, biological fluids or the whole body. Advances in proteomics have been largely driven by the development of mass spectrometry and two-dimensional electrophoresis (2DE). The analysis and quantification of proteins has been revolutionized by MS-based methods and, recently, these have been adapted for high-throughput analyses of thousands of proteins in cells or body fluids (Hein, Sharma, Cox, & Mann, 2013; Selevsek et al., 2015). The functions of a large fraction of proteins are mediated by post-translational modifications such as proteolysis, glycosylation, phosphorylation, nitrosylation, and ubiquitination (Beck et al., 2006; Mann & Jensen, 2003). Therefore, proteomics can reveal most translational, translational and post-translational changes. Recently, a shotgun proteomic strategy combined with high-accuracy mass spectrometry has been used to study a bacterial phosphor-proteome. The study revealed that phosphorylation of *A. baumannii* β-lactamase at the active site of the protein leads to an imipenem-sensitive phenotype, whereas the non-phosphorylated enzyme exhibits a high β-lactamase activity and provides resistance against imipenem (Lai et al., 2016; Lai et al., 2017). Zhou and others established that succinylation of different lysine residues in isocitrate lyase of *M. tuberculosis* is associated with resistance to different anti-bacterial compounds (Zhou, Xie, Yang, Zhou, & Xie, 2017).

Another useful aspect of proteomics is the identification of extracellular proteins that either could be secreted freely or confined within extracellular vesicles. Investigation of cellular proteomes and extracellular vesicles in *Pseudomonas aeruginosa* grown in biofilm or planktonic culture revealed that the development of antibiotic resistance in biofilms is

associated with modulation of both the cellular and vesicular proteomes (A. J. Park, Surette, & Khursigara, 2014). The classification of differentially expressed microbial proteome profiles could reveal specific signatures that occur in response to drugs and help prioritize the compounds being tested for their selectivity and specificity. One of the options for classification of the large data sets obtained using omics technologies is the construction of molecular networks and pathways (Anitha, Anbarasu, & Ramaiah, 2014; Villaveces, Koti, & Habermann, 2015; Wenzel & Bandow, 2011).

Metabolomic approaches can be targeted or untargeted; Targeted metabolomics is increasingly popular for studies of metabolic changes associated with antimicrobial resistance. Schelli *et al.,* have used HPLC on a HILIC (hydrophilic interaction chromatography) column in combination with mass-spectrometry to monitor metabolites in two isogenic *S. aureus* strains with differential susceptibility to methicillin that were treated with sub-lethal doses of ampicillin, kanamycin, and norfloxacin. The treatment resulted in a set of similar and divergent metabolic alterations involving the metabolism of purines, pyrimidines, and amino acids (Schelli, Zhong, & Zhu, 2017).

The targeted approach can be supplemented with untargeted LC-MS to identify further targets to address concerns about potentially missing metabolites. Untargeted LC-MS is a method of choice if unusual metabolites are expected, as in the case of chemical transformation of antimicrobials, or if the range of metabolites affected is difficult to predict. One version of untargeted mass spectrometry involves direct sample injection into a mass spectrometer, without the LC-separation step (Pinu & Villas-Boas, 2017; Zampieri et al., 2017). The popularity of untargeted LC-MS for metabolomics has been supported by the rapid development of efficient and user-friendly bioinformatics tools. Powerful and flexible bioinformatics platforms such as OpenMS, mzMine2, MAVEN, MetaboAnalyst2, and XCMS

ensure the efficient and comprehensive analysis of large amounts of data obtained using untargeted LC-MS (Baptista, Fazakerley, Beckmann, Baillie, & Mur, 2018).

Nuclear Magnetic Resonance (NMR) is a very attractive technique for metabolomics because it provides broad coverage of metabolites, does not require prior separation of the metabolites, and is non-destructive, so samples can be retained for other analyses. 1D-NMR and 2D-NMR are currently being used for high-throughput metabolomics studies. For example, 1D ^{1}H NMR metabolomics has been used to monitor intra- and extra-cellular metabolites of *E. coli* upon the exposure to ampicillin, carbenicillin, tetracycline, doxycycline, kanamycin, streptomycin, ofloxacin, cefalexin, and ciprofloxacin, revealing specific patterns of metabolites by intracellular fingerprinting and extracellular foot printing is a valuable technique for the identification of bacterial cell responses to a specific antimicrobial and for predicting the effects of combinations of antimicrobials (Hoerr et al., 2016).

Monitoring the metabolome during exposure to different antimicrobials may help to identify metabolic processes contributing to the global cell response to antimicrobials. Recent studies have suggested that antimicrobial drugs may significantly modulate antimicrobial susceptibility and resistance of the phenotype (Baptista, et al. 2018; Crusco et al. 2019). For example, Zampieri and others have found that resistance to three different antimicrobials may evolve much more rapidly on glucose than on acetate, because of greater metabolic plasticity during respiro-fermentative metabolism on glucose than purely respiratory metabolism on acetate (Zampieri et al., 2017).

Additionally, metabolomics in combination with modelling has been used extensively to study metabolite flux. (Dettmer, Aronov, & Hammock, 2007; Madsen, Lundstedt, & Trygg, 2010; Mathew & Padmanaban, 2013; Zampieri et al., 2018). Crucially, metabolomics reflects the summation of transcriptional and post/translational control as well as such as allosteric control

of biochemical pathways. It is therefore perhaps the most informative 'omics' level in terms of describing the functions of a cell. In summary, a knowledge of the metabolic responses of both micro- and macro-organisms to biotic and abiotic stress factors, including antimicrobials, may contribute significantly to the development of new antimicrobials (Aros-Calt et al., 2015; Belenky et al., 2015).

This study assessed if the HB5d/e3 (A3) and HB5d/e5 (A5) fraction of *Dryopteris crassirhizoma* acts differently from established antibiotics on MRSA using metabolomics. Another aim is to use metabolomics to define the mode of action of the bioactive molecule(s) in HB5d/e3 (A3) and HB5d/e5 (A5).

6.2. MATERIALS

Chemicals:

HB5d/e5 (A5), Hb5d/e3 (A3), were extracted from *D. crassirhizoma* (See Chapter 5). Chloramphenicol (CH), Gentamicin (G), Levofloxacin (L), Nalidixic acid (N), Streptomycin (S), Vancomycin (V), were obtained from Sigma-Aldrich. The established antibiotics have accepted mode of actions (Table IX). Glutaraldehyde and sodium cacodylate (Agar Scientific Ltd, UK), ultra-low gelling temperature agarose solution (Sigma Aldrich Cat. No. A-5030), syringe filter (Whatman Ltd, UK), % osmium tetroxide (Agar Scientific Ltd), Hettich Mikroliter D-7200 micro-centrifuge, AMB stain Azur II & Methylene blue (Sigma Aldrich Ltd, UK), Butvar B98 polymer (Agar Scientific), uranyl acetate (Agar Scientific).

6.3. METHODS

Determination of *in vitro* antibacterial activity and dosage

To access the concentration of antibiotics required to inhibit the bacterial growth by 50%, a micro dilution of all antibiotics in a bacterial culture of OD600 0.6 was performed. The optical density OD600 was measured after 6 h of treatment (Baptista, Fazakerley, et al., 2018).

Examination of bacterial samples by transmission electron microscopy (TEM):

Bacterial cells were cultured with A3 and A5 at concentrations which had an MIC_{50} at 6 h and also higher concentration (equivalent to an MIC_{90} at 24 h) and an untreated control. The cultures were samples at 6 h which represented mid-exponential phase. At 6 h, 1 mL of the culture was collected and centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and cell pellets used for fixing. The whole contents of the microcentrifuge tubes were mixed

by vertexing with 1 mL of a primary fixative consists of 2.5 % glutaraldehyde in 0.1M sodium cacodylate at pH 7.2 (Agar Scientific Ltd, UK). These are be kept in the fridge until use.

Then, samples were centrifuged and the supernatant discarded following with the cells were re-suspended in fresh fixative. After 30 min fixation, the samples were centrifuged, and the supernatant discarded. The pellets were re-suspended in another 1ml of fresh fixative as above and left for 30 minutes, the previous step was repeated but they were re-suspended in 1ml 0.1 M sodium cacodylate wash buffer pH at 7.2 for 30 min. The samples were centrifuged, and the supernatant discarded. They were re-suspended in 1 mL of a secondary fixative consisting of 1 % osmium tetroxide made up in 0.1 M sodium cacodylate buffer pH 7.2. After 30 min, the samples were centrifuged, and the supernatant was carefully discarded. It was replaced with a quick rinse in 1 mL of 1 M sodium cacodylate wash buffer as above. After 5 min rinse, the samples were centrifuged, and the supernatant discarded. The pellets were re-suspended in another 1 mL of wash buffer. The samples were centrifuged, and the supernatant discarded. The samples were re-suspended in 100 µL 2 % ultra-low gelling temperature agarose solution (Sigma Aldrich) was made up in ultra-pure H_2O at 25^{o}C and placed in a refrigerator to gel at 4^{o}C.

After gelling overnight, the agarose containing the bacteria was cut from the microcentrifuge tubes with single-sided razor blades and transferred into 1 mL wash buffer in 5 mL glass vials with push-on lids (Scientific Glass Laboratories Ltd) at 4 ^{o}C. After 30 min the gelled agarose pellets were placed in fresh wash buffer. The samples were then passed through a sequential ethanol series of 30, 50, 70, 95% (v/v) and finally three changes of 100 %. Each step lasted for at least a 1 h. The samples were transferred to a 1:2 mixture of ethanol to LR White - Hard Grade (London Resin Company, UK) resin then a 2:1 mixture of ethanol to resin and finally 100 % resin overnight @ 4^{o}C. The resin was removed and replaced with fresh resin and later

that day the samples were then placed in size 4 gelatine moulds (Agar Scientific, UK), filled up with fresh resin and polymerised overnight in an oven at 60 ºC.

Sections (2µm thick) which contained the bacteria were cut and dried down on drops of 10 % ethanol on glass microscope slides. Sections were stained with AMB stain (Azur II & Methylene blue, both Sigma Aldrich Ltd, UK), and photographed using a Leica DM6000B microscope. Ultrathin 60-80 nm sections were then cut on a Reichert-Jung Ultracut E Ultramicrotome with a Diatome Ultra 45 diamond knife and collected on Gilder GS2X0.5 3.05 mm diameter nickel slot grids (Gilder Grids, Grantham, UK) float-coated with Butvar B98 polymer films. All sections were double stained with uranyl acetate and Reynold's lead citrate (TAAB Laboratories Equipment Ltd, Aldermaston, UK) and observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV. The resulting images were photographed using Carestream 4489 electron microscope film (Agar Scientific, UK) developed in Kodak D-19 developer for 4 min at 20 °C, fixed, washed and dried according to the manufacturer's instructions. The resulting negatives were scanned with an Epson Perfection V800 film scanner and converted to positive images.

Metabolomics

Sample preparation: The bacterial incubation constant shaking at 200 rpm at 37 °C. Six biological replicates of each isolate treated with antibiotic and the untreated control group were independently cultured. The sample preparation was done with some alteration from Baptista, et al. 2018. All samples were collected during mid-exponential growth phase. A 3 mL aliquot of bacterial culture (OD600 at time 0 h was 0.6) was harvested at 0, 2, 4 and 6 h after the treatment with the respective antibiotic. The samples were centrifuged at 10 °C at 4500 rpm. The yielded pellet was then resuspended with 3 mL of cold saline solution (0.85 % NaCl in H_2O w/v) and OD was noted, with consequent centrifugation at 10 °C at 4500 rpm. The

samples were stored at −80 °C after the cellular metabolism was rapidly quenched with liquid N_2. After thawing, the samples were centrifuged at 10 °C at 4500 rpm. The yielded pellet was then washed with 4 mL of cold saline solution (0.85 % NaCl in H_2O w/v) with consequent centrifugation at 10 °C at 4500 rpm. All samples were adjusted to an OD600 of 1. The samples were centrifuged and 70 µL of a chloroform/methanol/water (1: 3: 1) mixture was added. The extractions involved four freeze-thaw cycles with periodic vortexing. After final centrifugation at 4500 rpm, 60 µL of the particle-free supernatant was transferred into a micro centrifuge tube. An additional extraction with 50 µL of chloroform/methanol/water (1: 3: 1) was done and the new supernatant, after final centrifugation, was combined with the supernatant from the first extraction. From this mixture, 50 µL were transferred into an HPLC vial containing a 0.2 mL flat-bottom micro insert for FIE-HRMS analysis (See 2.2).

***Metabolomics data annotation*:** MetaboAnalyst 4.0. - Statistical analysis (Chong et al., 2018) was used to perform principal component (PCA). MetaboAnalyst 4.0 - MS peaks to pathway(Chong et al., 2018) was used to identify metabolites (tolerance = 3 ppm) and significant affected metabolic pathways (model organism = *S. aureus*). MetaboAnalyst pathway identification is based on mummichog, an algorithm able to predict biological activity directly from mass spectrometry data, avoiding the *a priori* identification of metabolites (S. Li et al., 2013). Mummichog plots all possible matches in the metabolic network and then looks for local enrichment, providing reproduction of true activity, as the false matches will distribute randomly (S. Li et al., 2013). Examples of key-metabolites in local enrichment were analysed for significant difference (t-test) between control and treatment using Microsoft Excel.

6.4. RESULTS

In this study, intracellular metabolic profiles were exploited to investigate the impact of antibiotic compounds with different known cellular targets on the metabolome of *Staphylococcus aureus* USA300 in comparison to the anti-MRSA compounds obtained from *D. crassirhizoma.* Alterations within the metabolic pool represent the physiological status of the bacteria as the result of the adaptive cascades comprising both the transcriptome and proteome levels. Moreover, metabolites with regulatory and signaling functions act as important links between the metabolome, gene transcription, and protein biosynthesis. Thus, to understand the mode of action of A3 and A5 its metabolic response of MRSA was compared to a range of antibiotics, which were selected based on their different targets (Table IX).

Table IX: Antibiotic used and their respective targets

Antibiotic	Targets
Chloramphenicol (CH),	RNA - Binds 50S subunit (L16) or rRNA inhibiting protein synthesis.
Gentamicin (G),	RNA - aminoglycoside - Binds 30S subunit (S12) of rRNA misreading mRNA and incorrect amino acid insertion.
Levofloxacin (L),	DNA - 4-quinolone - Inhibition of the enzyme topoisomerase II (DNA gyrase) & IV preventing DNA replication and transcription (Drlica & Zhao, 1997).
Nalidixic acid (N),	DNA - 4-quinolone - Inhibition of the enzyme topoisomerase II (DNA gyrase) & IV preventing DNA replication and transcription (Drlica & Zhao, 1997).
Streptomycin (S)	RNA - Binds 30S subunit (S12) of rRNA misreading mRNA and incorrect amino acid insertion.
Vancomycin (V),	Cell Wall - Prevents incorporation of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) peptide subunits from being incorporated into the peptidoglycan matrix. In addition, altering bacterial-cell-membrane permeability and RNA synthesis.

Standardisation of antibiotic treatments: This mode of action study included comparison with other antibiotics which have difference MICs (Table X). Antibiotic treatments need to be standardized so that each antibiotic is having an equal suppressive effect on the bacterial growth over 6 h. In addition to the standardisation of treatments, a more concentrated bacterial

culture was required for metabolomics as $1x10^8$ CFU/mL was found to be insufficient concentration of metabolites after extraction for metabolomic analysis. Therefore, initial experiments were carried out to calculate the antibiotic concentrations required to suppress bacterial growth by 50% at 6 h following inoculated (MIC_{50}) and also aby 90% at 24 h following inoculation (MIC_{90}).

Table X: Standardisation of antibiotics based on MIC.

Concentration of antibiotic required to inhibit growth of USA300 MRSA at 24hours and by 50% over 6 hours at a bacterial concentration of $1x10^8$ CFU/mL. Note: HB5d/e5 (A5), Hb5d/e3 (A3), chloramphenicol (CH), Gentamicin (G), levofloxacin (L), nalidixic acid (N), streptomycin (S), vancomycin (V).

Compounds	MIC_{90} 24h (µg/mL)	MIC_{50} 6h (µg/mL)
A5	12.5	0.1
A3	6.25	0.49
L	0.17	0.49
S	125	0.166
V	1.33	0.33
CH	3.90	1.95
N	31.25	7.8125
G	7.81	0.49

Transmission electron microscopy: Phenotypic changes in cells after drug treatment can give a broad indication of the possible mode of actions. Therefore, TEM was initially used to visualise the effect of A3 and A5 on MRSA cells. Two different concentrations were assessed. The lower concentration of both A3 and A5 was the MIC_{50} value at 6h (0.49 µg/mL and 0.1 µg/mL respectively) and the higher concentration was the MIC_{90} value at 24h (6.25 µg/mL and

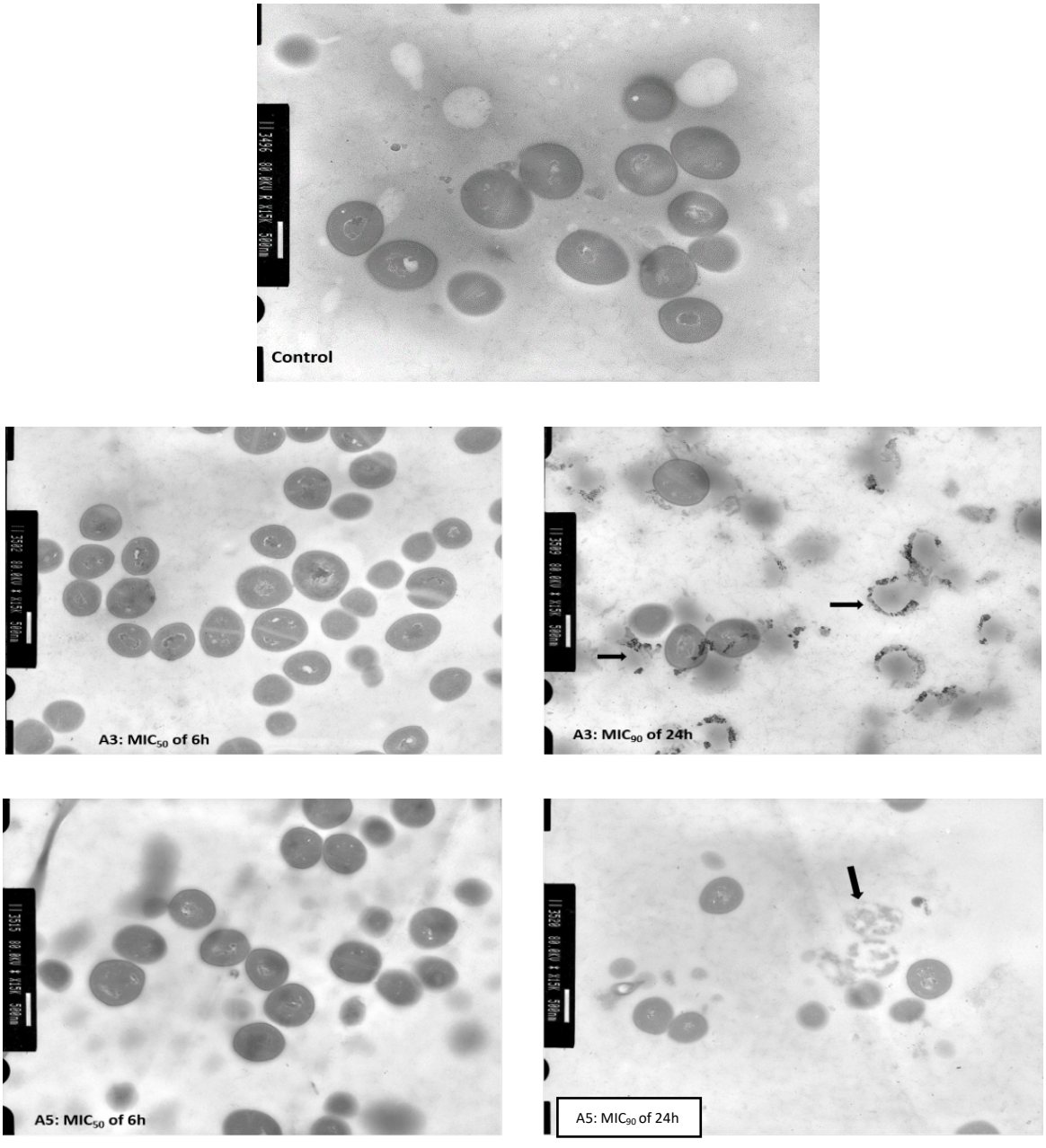


Figure 6.3: Transmission electron microscope images of MRSA:USA 300 treated with two different concentration of A3 and A5

Left: low concentrations: MIC_{50} of 6h, right: high concentration: MIC_{90} of 24h; Arrow shows the cell membrane disruption and lysis.

12.5 µg/mL respectively) (Table X). These two concentrations were added individually to exponentially growing MRSA cultures and the cells were harvested after 4 h. It should be noted that this would result in microbial populations where a proportion of cells would still be viable. Another set with no antibiotics containing cells from the same culture and growth time was treated as a control. TEM of the control MRSA showed the expected rounded cells (Figure 6.3). At the lower concentrations of A3 (MIC_{50}) there was no appreciable difference to the controls and there was evidence of cell division occurring in this sample as evidenced by the formation of internal septa. Increasing the concentration to MIC_{90} had clear effects with no sign of septa formation but instead they were signs of cell membrane degradation as indicted by the electron-dense structures linked to the outer envelope (Figure 6.3, arrowed). Considering A5, the (MIC_{50} value) had little observable effect on cellular structure. However, increasing the concentration to the MIC_{90} value resulted in complete rupture of some cells (Figure 6.3, arrowed).

Metabolomics: Using the antibiotic concentrations required to suppress 50 % of MRSA growth at 6 h (Table X), a series of cultures were initiated treatment with these concentrations along with untreated controls. Samples were harvested at 0, 2, 4 and 6 h for the treated and control and prepared for metabolomic assessment. Metabolites derived from treated vs control MRSA were profiled by using FIE-HRMS in negative and positive ionization modes.

Unsupervised PCA indicated that the MRSA metabolome following treatment with fraction A3 at 2, 4 and 6 h was quite distinct from those of established antibiotics considering their mode of action (Figure 6.5). It was notable that the metabolomes of the established antibiotics clustered together and were therefore fundamentally similar, irrespective of their different models of action. This did not arise from the MRSA cells being all dead, as the concentrations of antibiotic only suppressed bacterial growth by 50 % at 6 h (Table X). A similar examination

of the MRSA metabolome was carried out following treatment with A5, at time points (Figure 6.5).

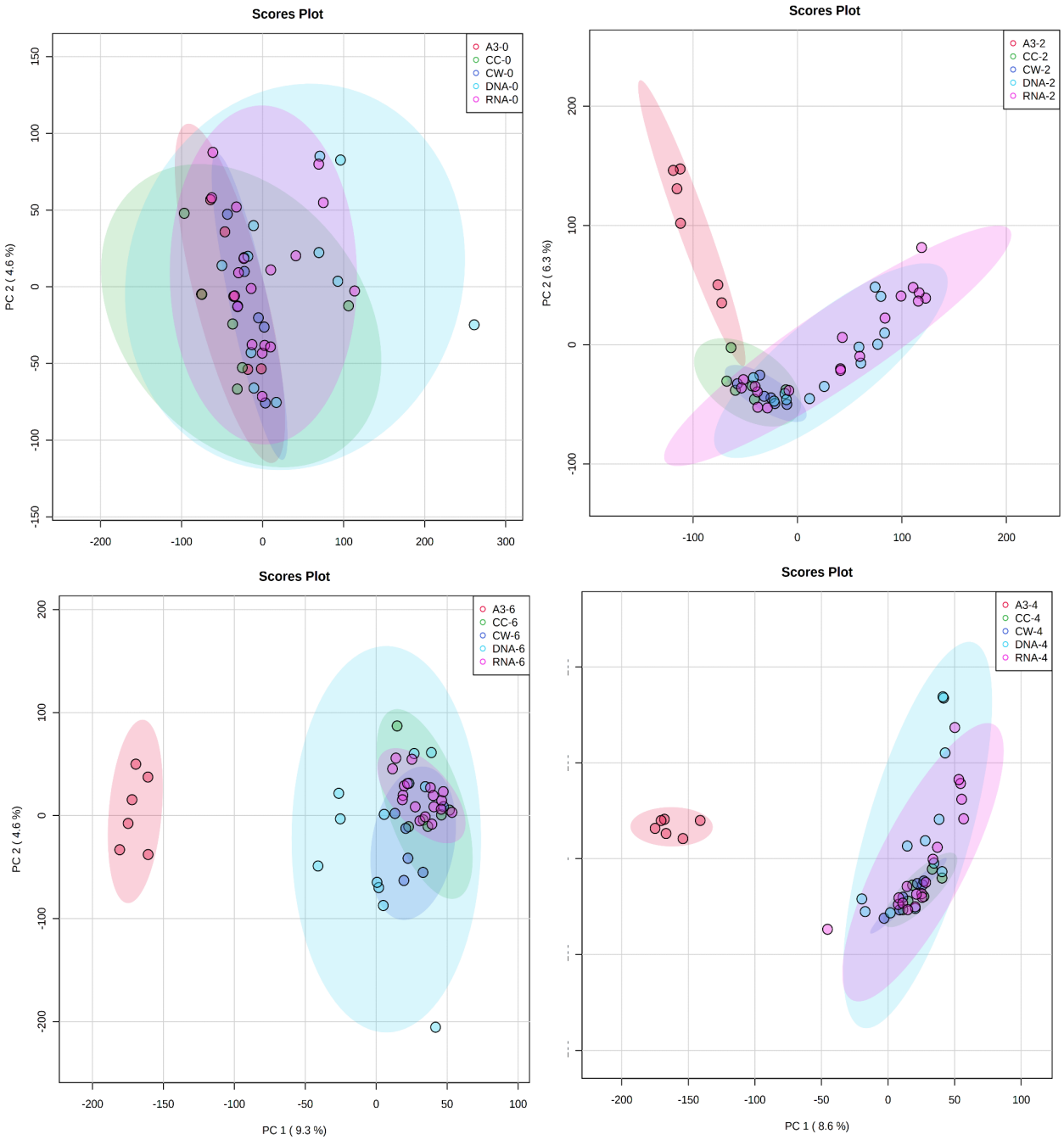


Figure 6.4: Principal component analysis (PCA) of antibiotic treated MRSA metabolomes.

PCA score plots (95% confidence interval illustrated, clear outliers removed) of normalized m/z intensities of metabolites extracted from MRSA treated with A3 and compared to control bacteria (CC) and to bacteria treated with antibiotics with similar mechanism of action, grouped into those with activity on cell wall (vancomycin, CW), on DNA (Levofloxacin and Nalidixic acid) and on RNA (Chloramphenicol, Gentamicin and Streptomycin) for both positive and negative mode. for time points 0,2,4 and 6 h. Plots indicate metabolome differences between treatment groups based on metabolite features detected by flow infusion electrospray high-resolution mass spectrometry (FIE-HRMS).

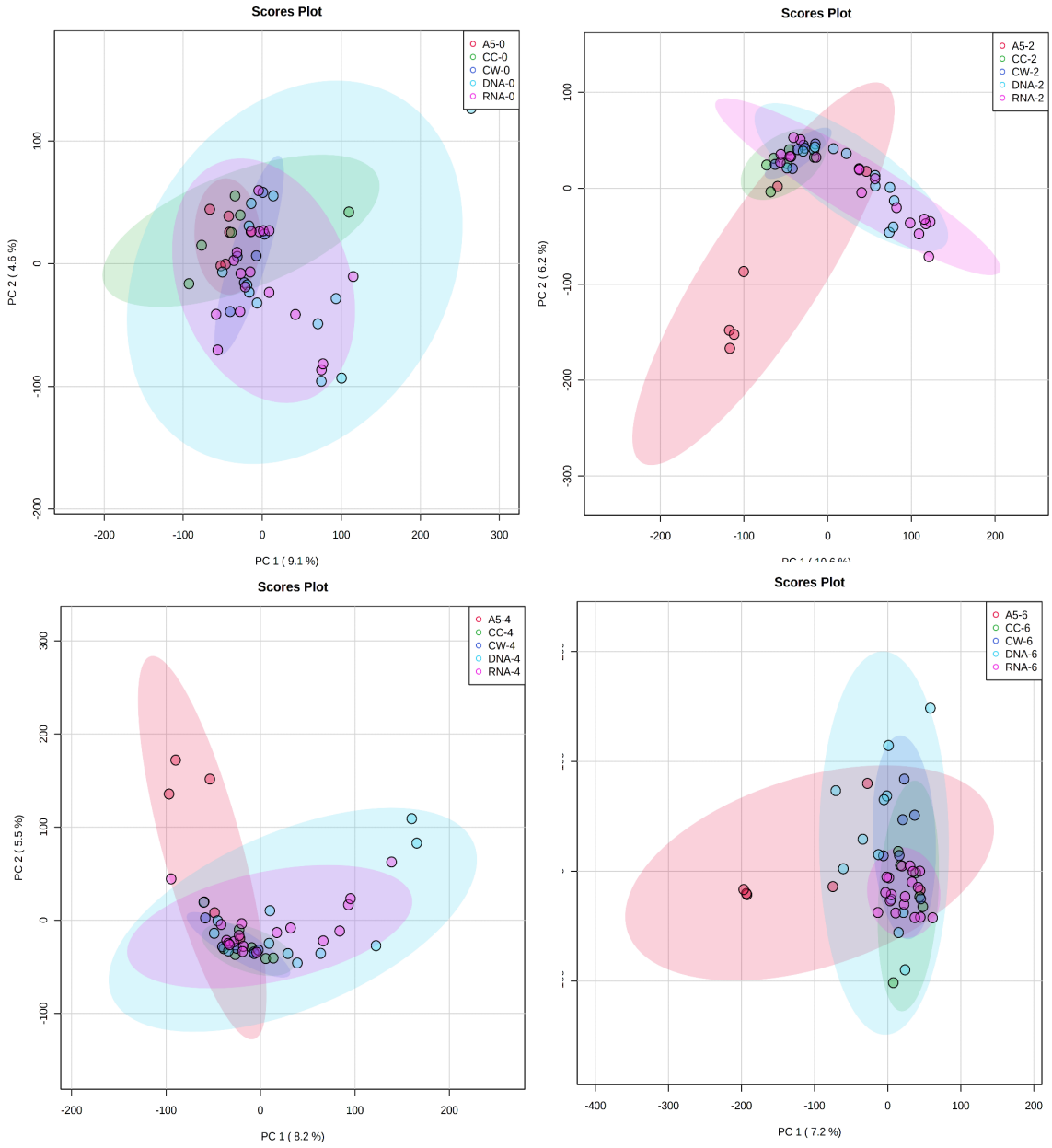


Figure 6.5: Principal component analysis (PCA) of antibiotic treated MRSA metabolomes.

PCA score plots (95% confidence interval illustrated, clear outliers removed) of normalized m/z intensities of metabolites extracted from MRSA treated with A5 and compared to control bacteria (CC) and to bacteria treated with antibiotics with similar mechanism of action, grouped into those with activity on cell wall (vancomycin, CW), on DNA (Levofloxacin and Nalidixic acid) and on RNA (Chloramphenicol, Gentamicin and Streptomycin) for both positive and negative mode. for time points 0,2,4 and 6 h. Plots indicate metabolome differences between treatment groups based on metabolite features detected by flow infusion electrospray high-resolution mass spectrometry (FIE-HRMS).

Further investigations aimed to assess the potential of metabolomics to indicate mode of actions of established antibiotics. Thus, the metabolites significantly different between control and

treated with the listed antibiotics at different time points were mapped on to KEGG pathway using the "MS peaks to pathway" function on MetaboAnalyst 4.0.

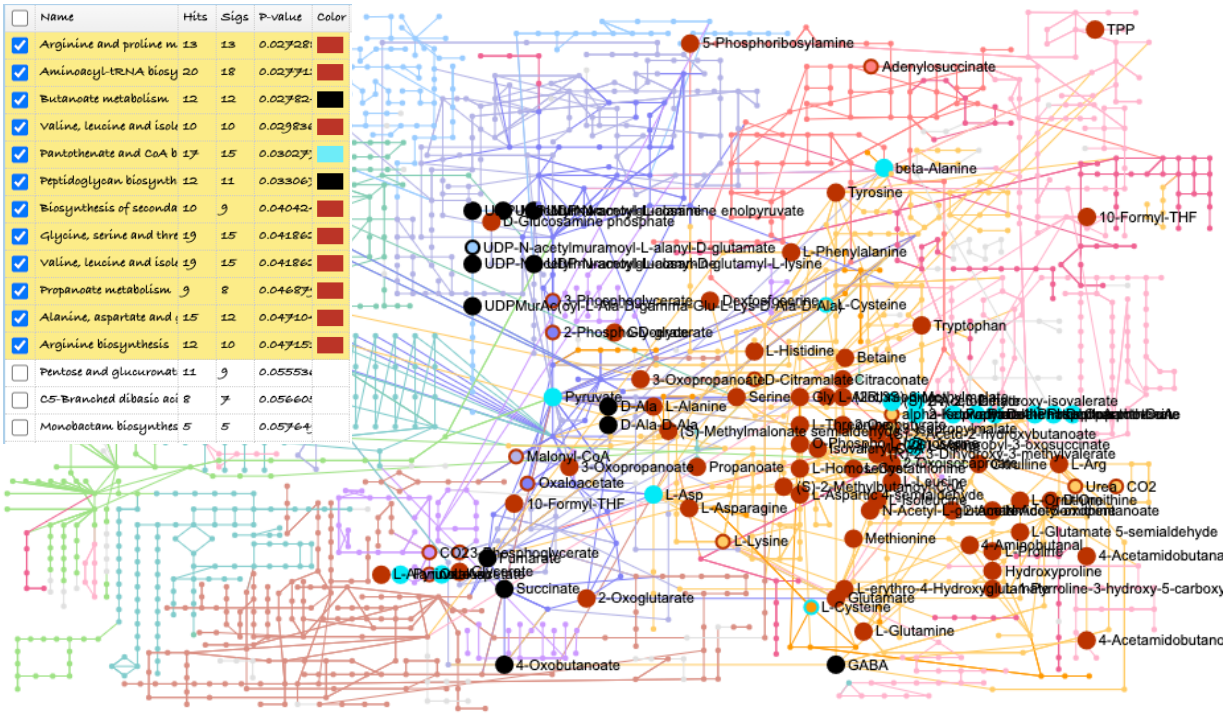


Figure 6. 6: MRSA metabolic networks affected by Chloramphenicol.

All the significantly affected pathway (p<0.15) based on the increasing p.value of negatively ionised metabolites. Amino acids are coloured in red; carbohydrate metabolism as black, and energy metabolism as light blue (when compared to the control MRSA samples**).**

Chloramphenicol is known to block peptidyl transferase activity by hindering the binding of tRNA to the A site (Moazed & Noller, 1987). Figure 6. 6 shows the vast majority of the significantly affected pathway involves amino acids and aminoacyl tRNA biosynthesis a clear indication of the effect of chloramphenicol on MRSA cells. It also shows cell dysregulation in pathways involved in general cellular homeostasis and energy metabolism such as butanoate metabolism and CoA biosynthesis.

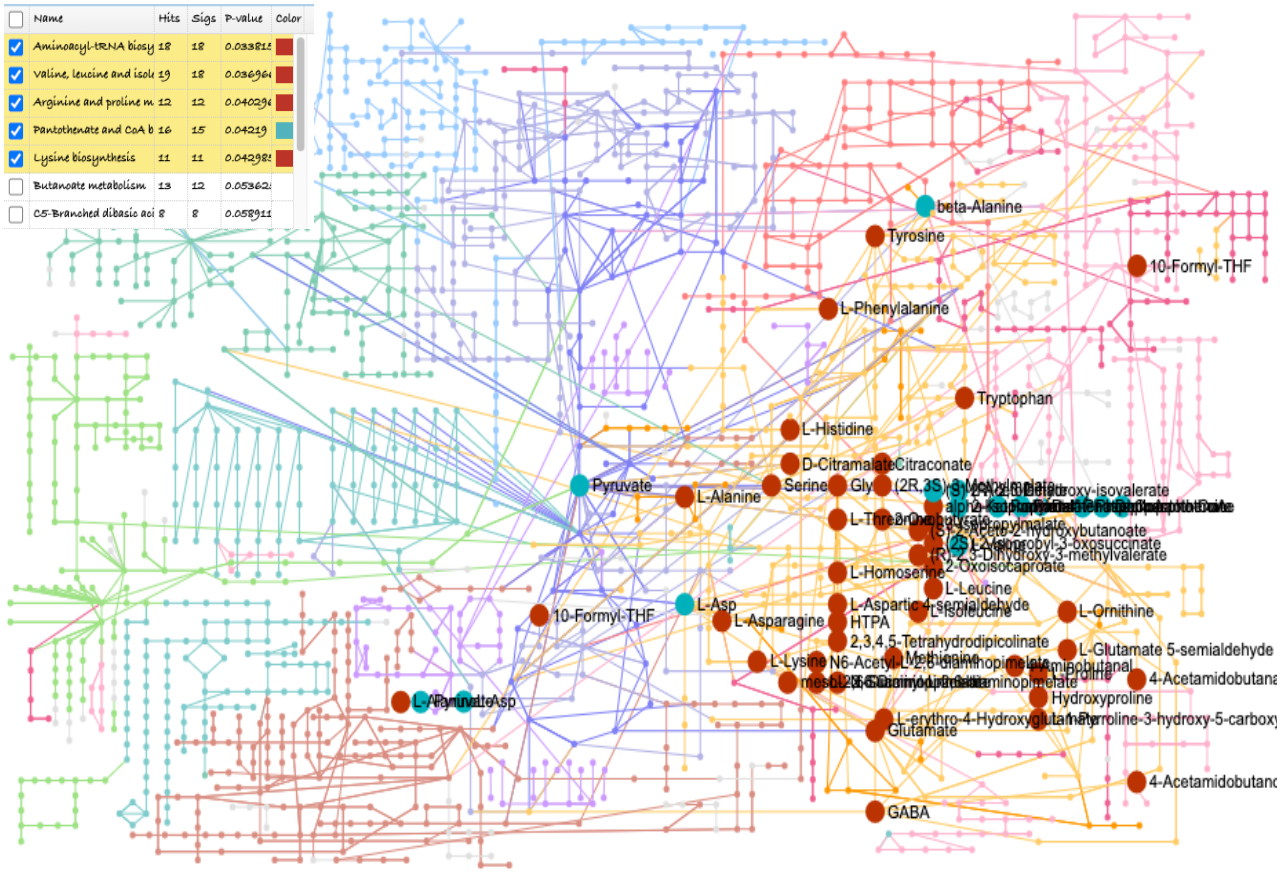


Figure 6. 7: MRSA metabolic networks affected by Streptomycin

All the significantly affected pathway (p<0.15) based on the increasing p.value value of negatively ionised metabolites. Amino acids are coloured in red; energy metabolism as light blue (when compared to the control MRSA samples**).**

Streptomycin causes mismatches between codons and anticodons, leading to faulty proteins that insert into, and disrupt, cytoplasmic membranes. Figure 6. 7 indicates the significant effect in the aminoacyl tRNA biosynthesis, amino acid pool along with lysine biosynthesis of MRSA when treated with streptomycin. As with chloramphenicol, these amino acid changes appeared to align with the established mode of action for streptomycin.

Gentamicin being in the same class of aminoglycoside antibiotic as streptomycin and chloramphenicol binds to the 16s rRNA at the 30s ribosomal subunit, disturbing the translation of mRNA(Beganovic et al., 2018) and, thus, leading to the formation of truncated or nonfunctional proteins. Significant effect on the amino acid pool of MRSA cells on treatment with gentamycin is clearly indicated in both negative and positive ionisation mode metabolites (Figure 6. 8 and Figure 6. 9). It was also noted in Figure 6. 9 that there was a change in nucleotide (purine) biosynthesis. Amino acids, particularly, glutamate and glutamine, feed into the *de novo* biosynthetic path for nucleotides and perturbations of this effect could be being observed here.

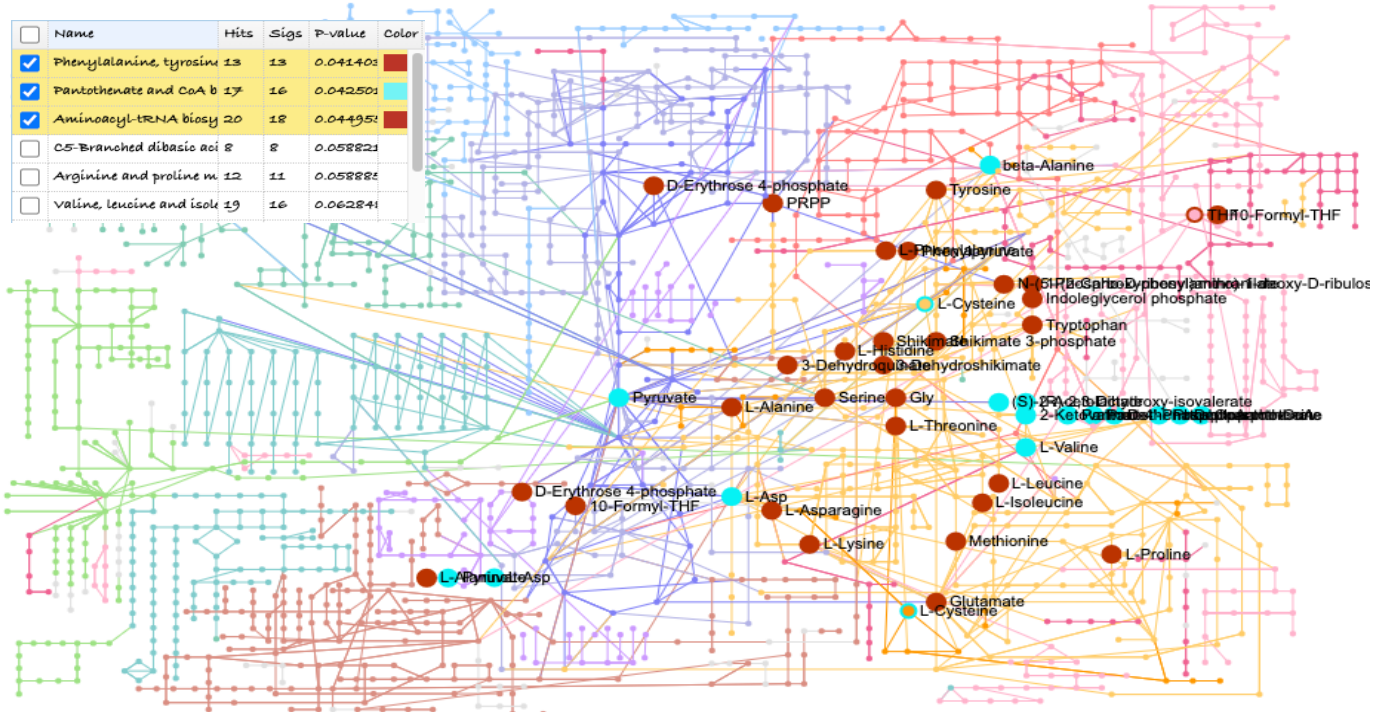


Figure 6. 8: MRSA metabolic networks affected by Gentamicin.

All the significantly affected pathway (p<0.15) based on the increasing p.value of negatively ionised metabolites. Amino acids are coloured in red and energy metabolism as light blue (when compared to the control MRSA samples**).**

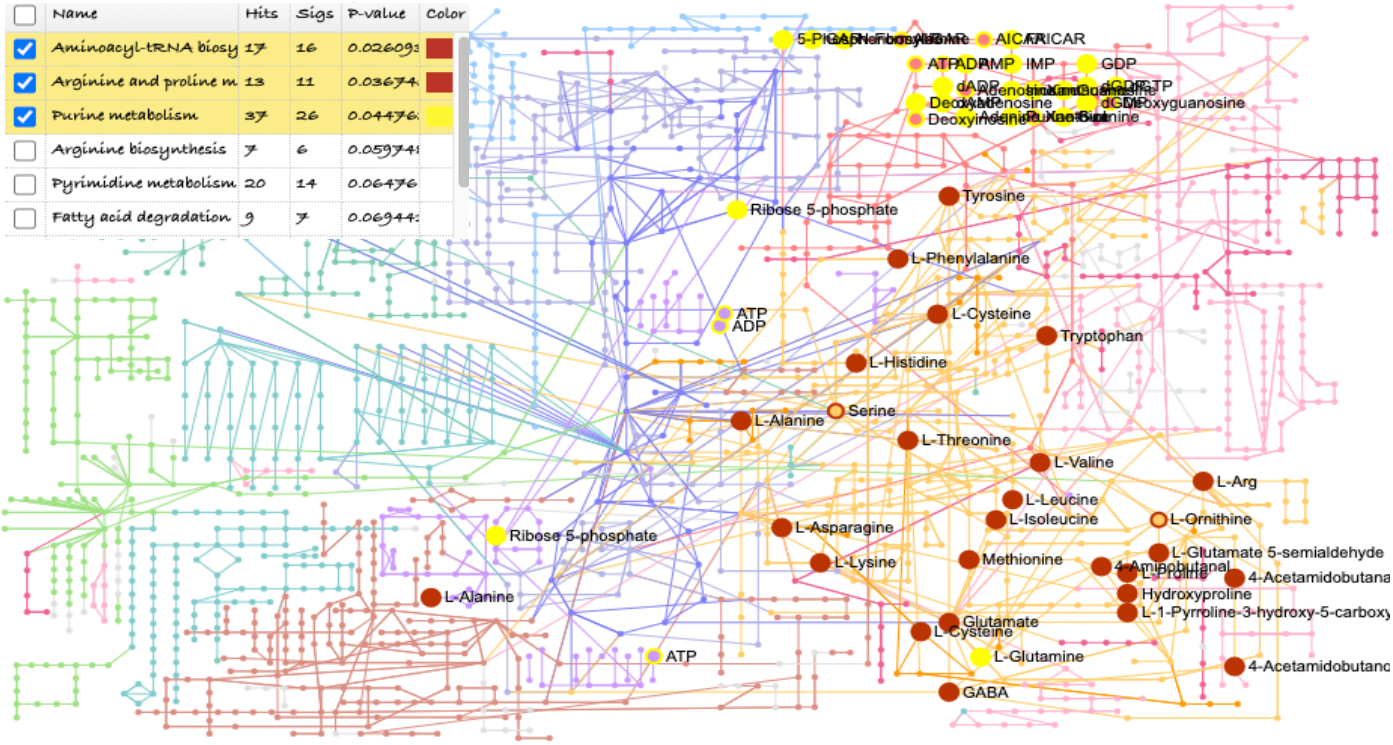


Figure 6. 9: MRSA metabolic networks affected by Gentamicin.

All the significantly affected pathway (p<0.15) based on the increasing p.value of positively ionised metabolites. Amino acids are coloured in red and Nucleic acid pathways as yellow (when compared to the control MRSA samples**).**

Nalidixic acid selectively inhibits the activity of bacterial DNA gyrase, blocking DNA replication. Figure 6.10 and Figure 6.11 shows the effect on nucleotides, amino acid pool, and cell dysregulation.

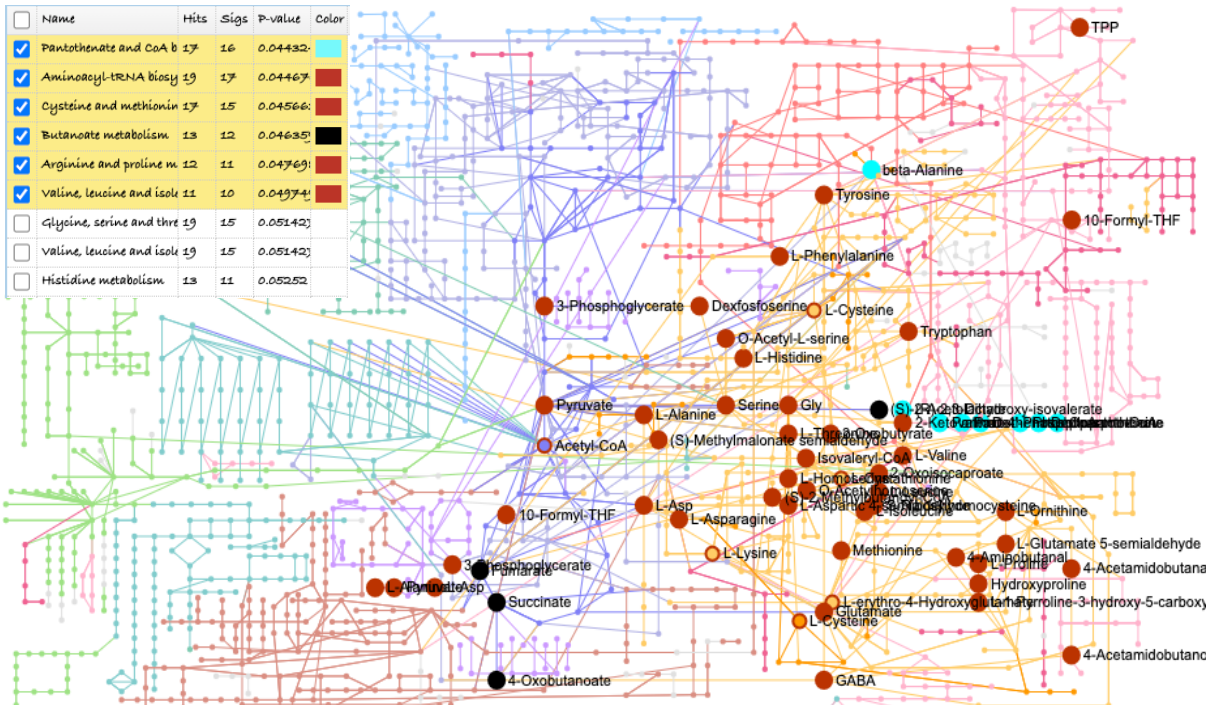


Figure 6.10: MRSA metabolic networks affected by Nalidixic acid.

All the significantly affected pathway (p<0.15) based on the increasing p.value of negatively ionised metabolites. Amino acids are coloured in red; carbohydrate metabolism as black, and energy metabolism as light blue (when compared to the control MRSA samples**).**

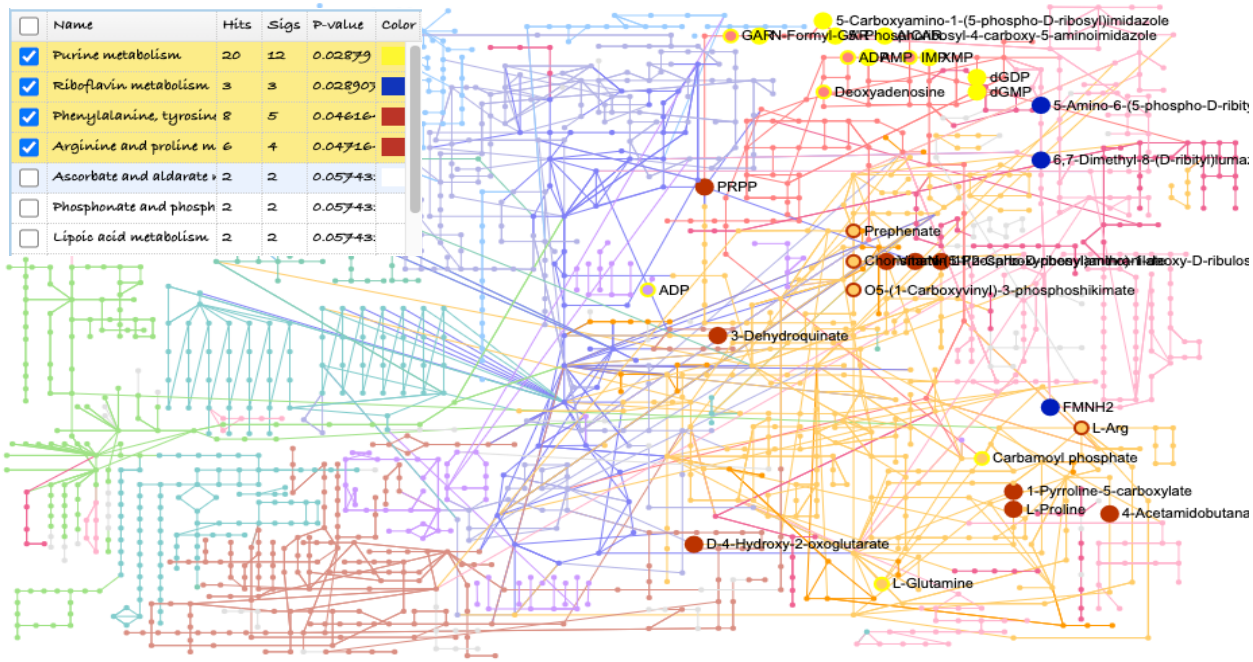


Figure 6.11: MRSA metabolic networks affected by Nalidixic acid.

All the significantly affected pathway (p<0.15) based on the increasing p.value of positively ionised metabolites. Amino acids are coloured in red; cofactor and vitamin metabolism as blue, and nucleotide metabolism as yellow (when compared to the control MRSA samples**).**

Levofloxacin a fluoroquinolone generally targets chromosome replication and in particular, DNA gyrase, which allows DNA unravelling before replication (Figure 6.12).

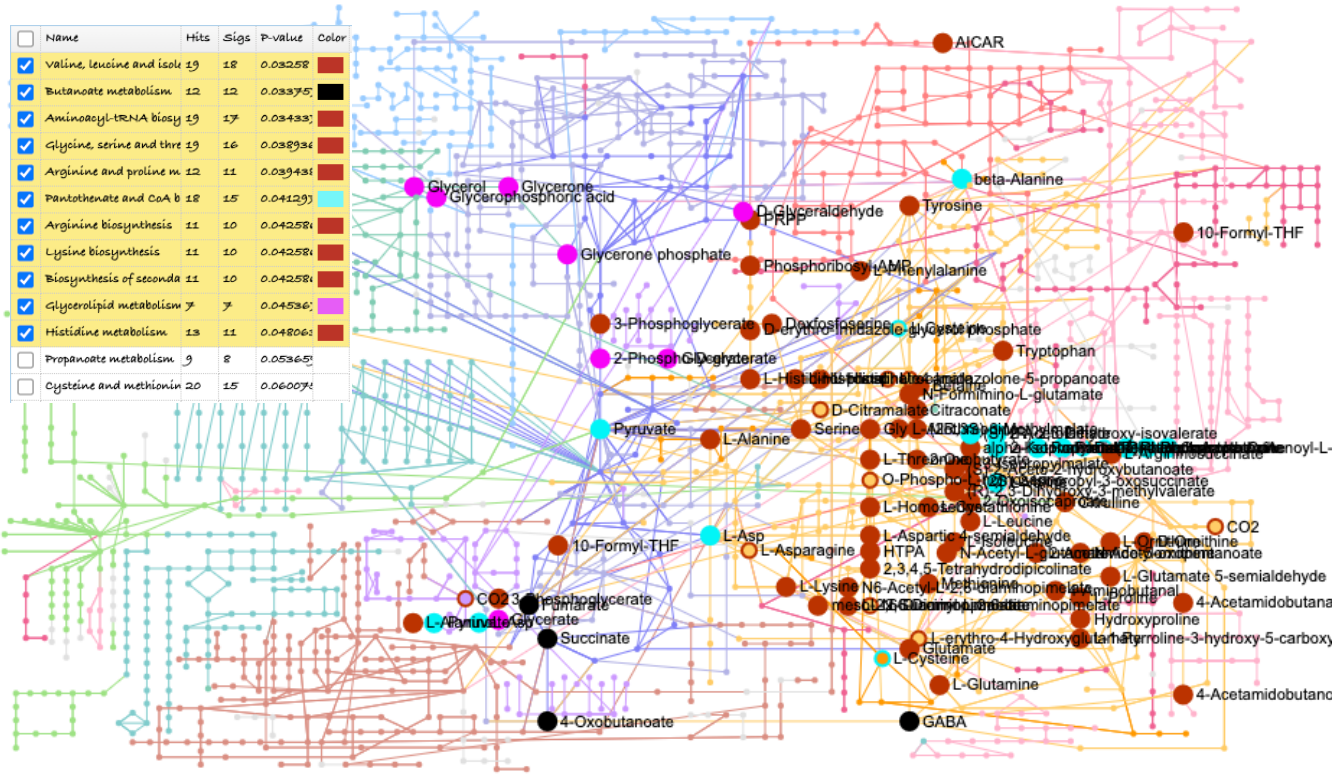


Figure 6.12: MRSA metabolic networks affected by Levofloxicin.

All the significantly affected pathway (p<0.15) based on the increasing p.value of negatively ionised metabolites. Amino acids are coloured in red; carbohydrate metabolism as black, fatty acid metabolism as pink and energy metabolism as light blue (when compared to the control MRSA samples**).**

A3 at different time points which significantly differ from control and other antibiotics. They are further mapped on the KEGG general metabolism map. It was notable that the vast majority of the significantly affected pathway involved glycolysis (Figure 6.13). To provide further insights into the effects of A3 on MRSA metabolism, the significantly differing metabolites were mapped on to heatmap in order to compare to untreated control are shown in Figure 6. 14.

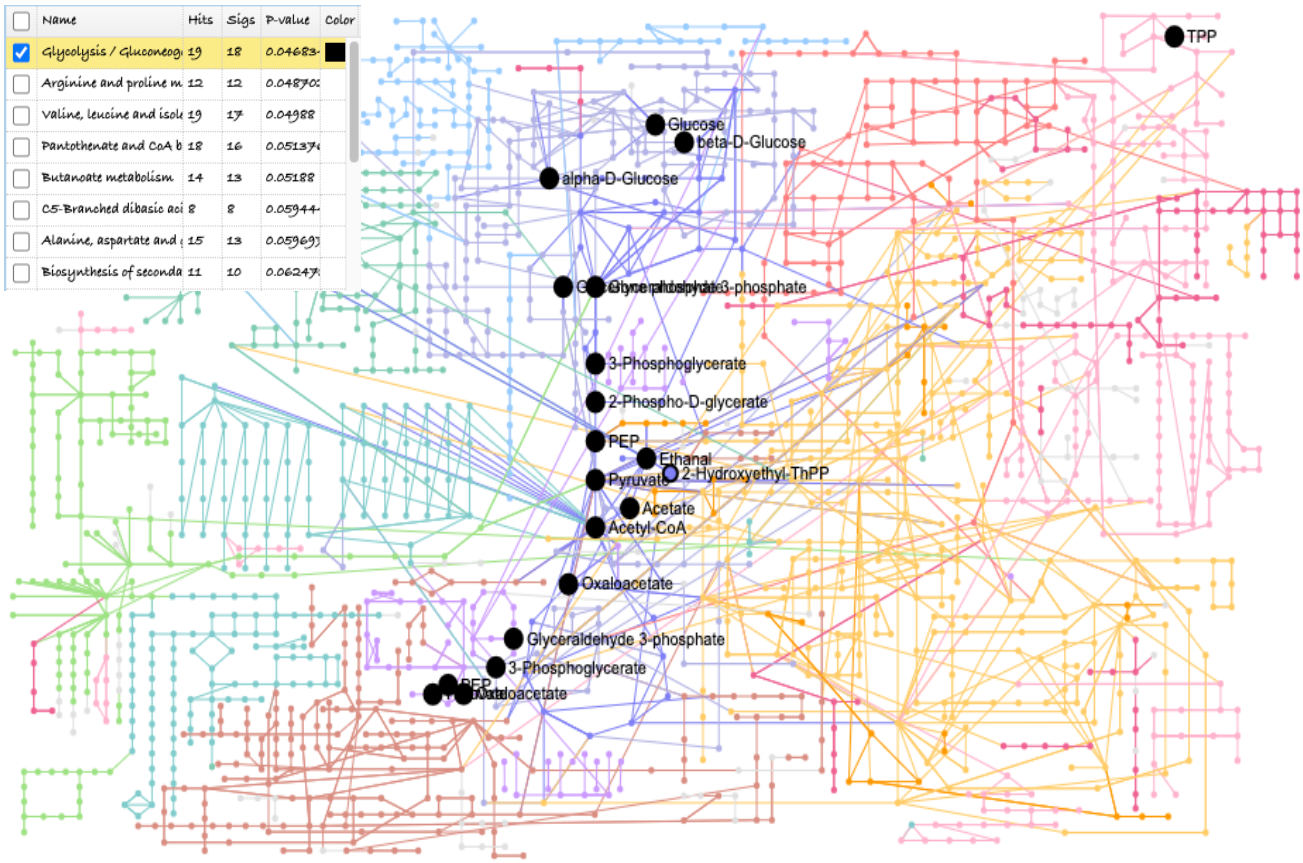


Figure 6.13: MRSA metabolic networks affected by A3.

All the significantly affected pathway (p<0.15) based on the increasing p.value of negatively ionised metabolites. Glycolysis pathway as black when compared to the control MRSA samples**.**

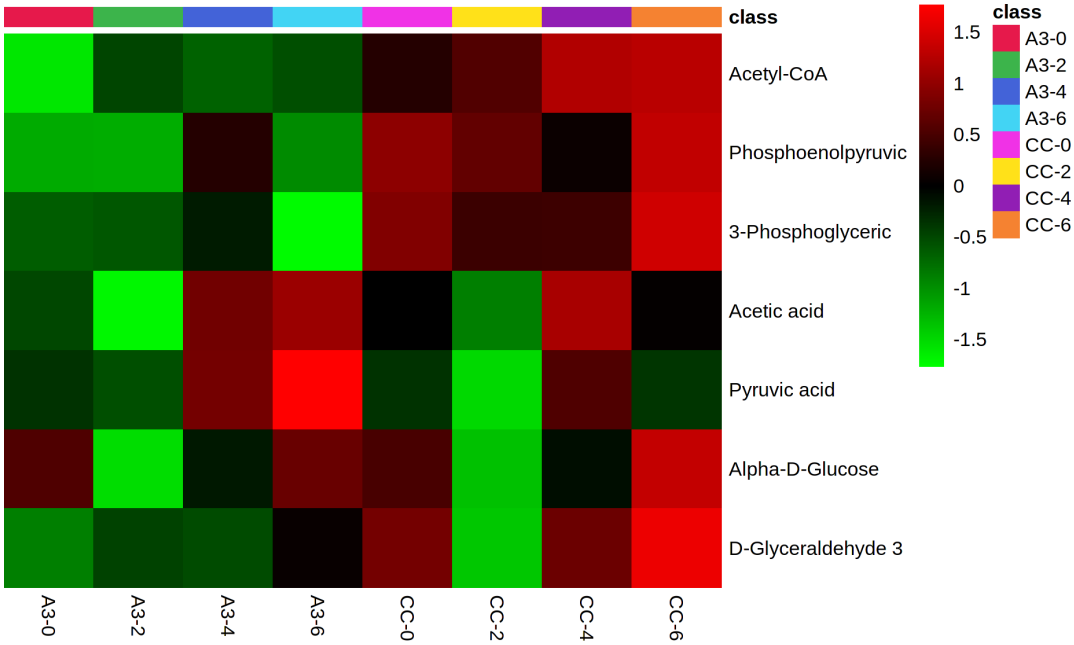


Figure 6. 14: Heatmap of all the significantly different metabolite changes of glycolysis and gluconeogenesis pathway happening at different time points in both treated (A3) and non-treated cells (CC).

A5 significantly effects variety of metabolic pathways including amino acids, fatty acid metabolism, nucleotide metabolism, carbohydrate metabolism and energy metabolism as observed in Figure 6. 15 and Figure 6. 16.

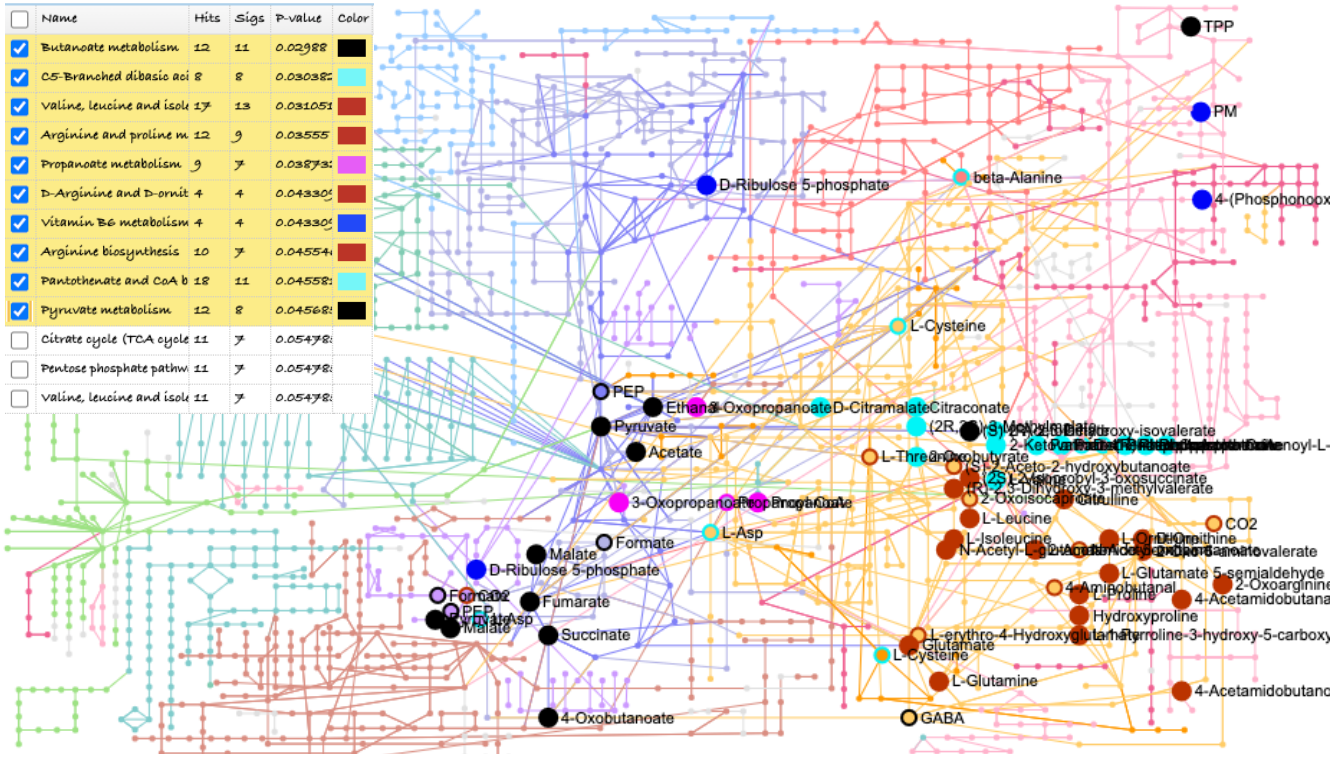


Figure 6. 15: MRSA metabolic networks affected by A5.

All the significantly affected pathway (p<0.15) based on the increasing p.value of negatively ionised metabolites. Amino acids are coloured in red; fatty acid metabolism as pink; cofactor and vitamin metabolism as blue; energy metabolism as light blue and carbohydrate metabolism as black (when compared to the control MRSA samples**)**

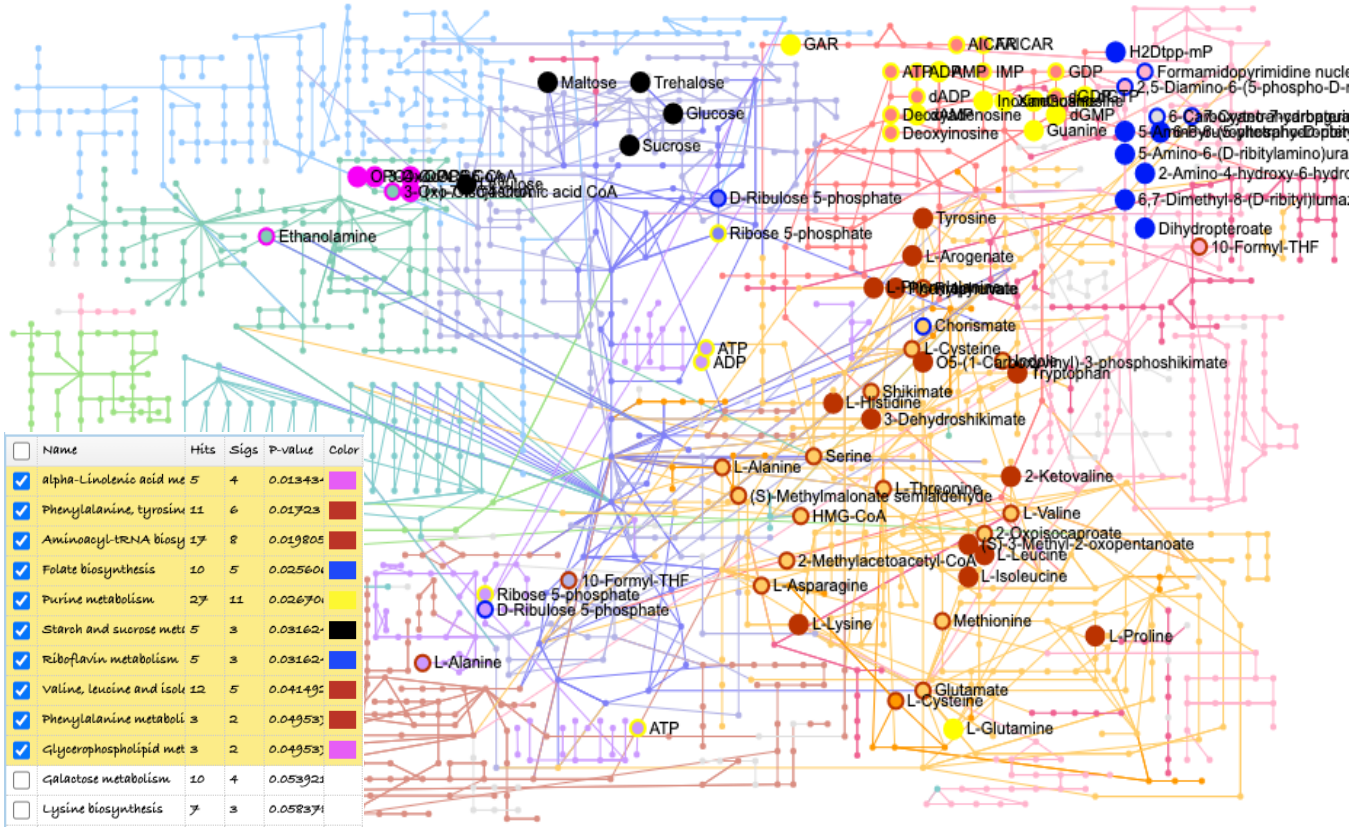


Figure 6. 16: MRSA metabolic networks affected by A5.

All the significantly affected pathway (p<0.15) based on the increasing p.value of positively ionised metabolites. Amino acids are coloured in red; fatty acid metabolism as pink; cofactor and vitamin metabolism as blue; nucleotide metabolism as yellow and carbohydrate metabolism as black (when compared to the control MRSA samples**).**

To provide further insights into the effects of A5 on MRSA metabolism, the significantly differing metabolites effecting different pathways were mapped on to heatmap in order to compare levels to untreated control are shown in Figure 6. 17 and Figure 6. 18.

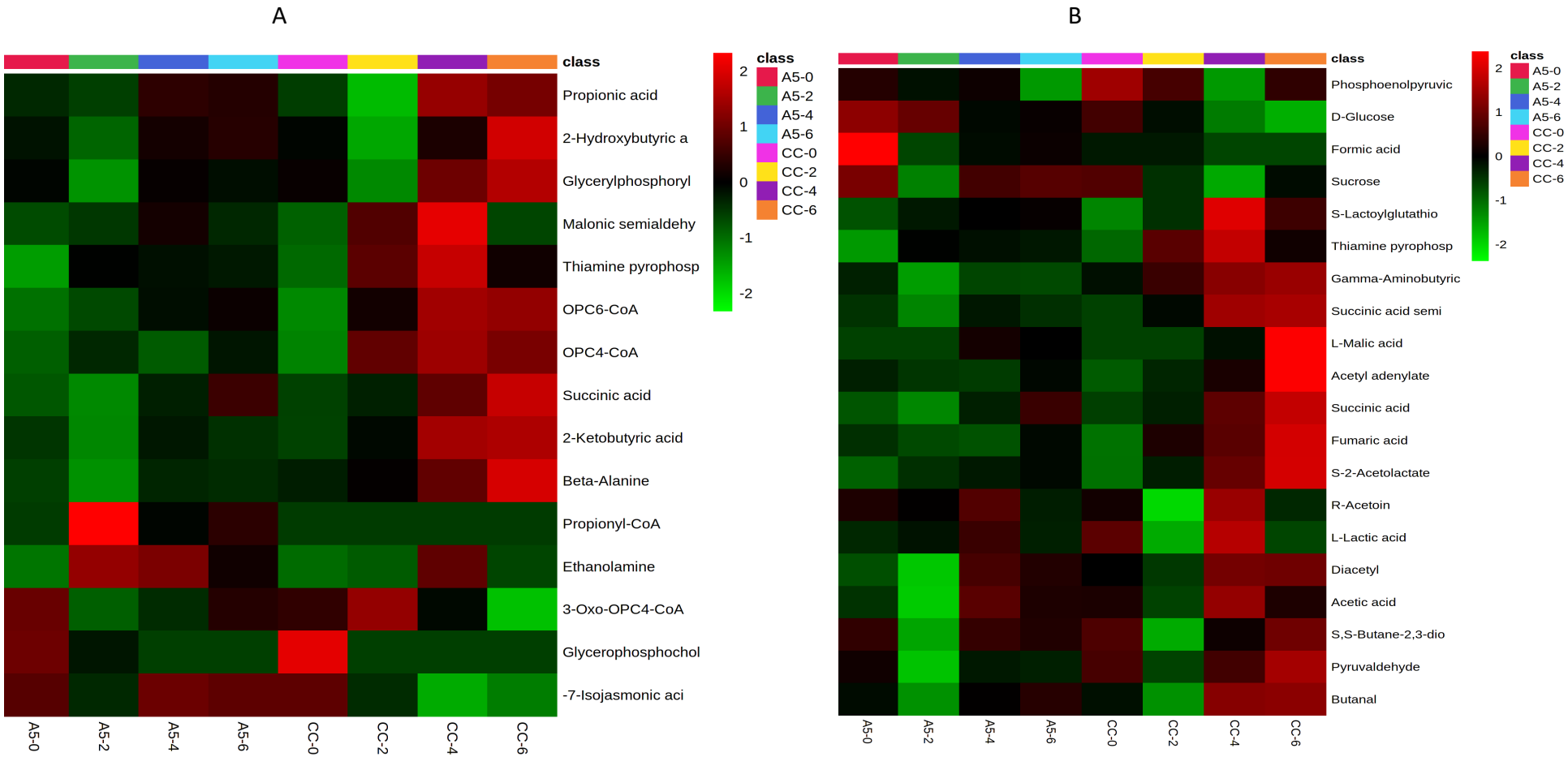


Figure 6. 17: Metabolite changes of MRSA treated with A5 in comparison to untreated cells

A: Heatmap of all the significantly different metabolite changes happening at different time points in both treated (A5) and non-treated cells (CC) in fatty acid metabolism; B: Heatmap of all the significantly different metabolite changes happening at different time points in both treated (A5) and non-treated cells (CC) in carbohydrate metabolism.

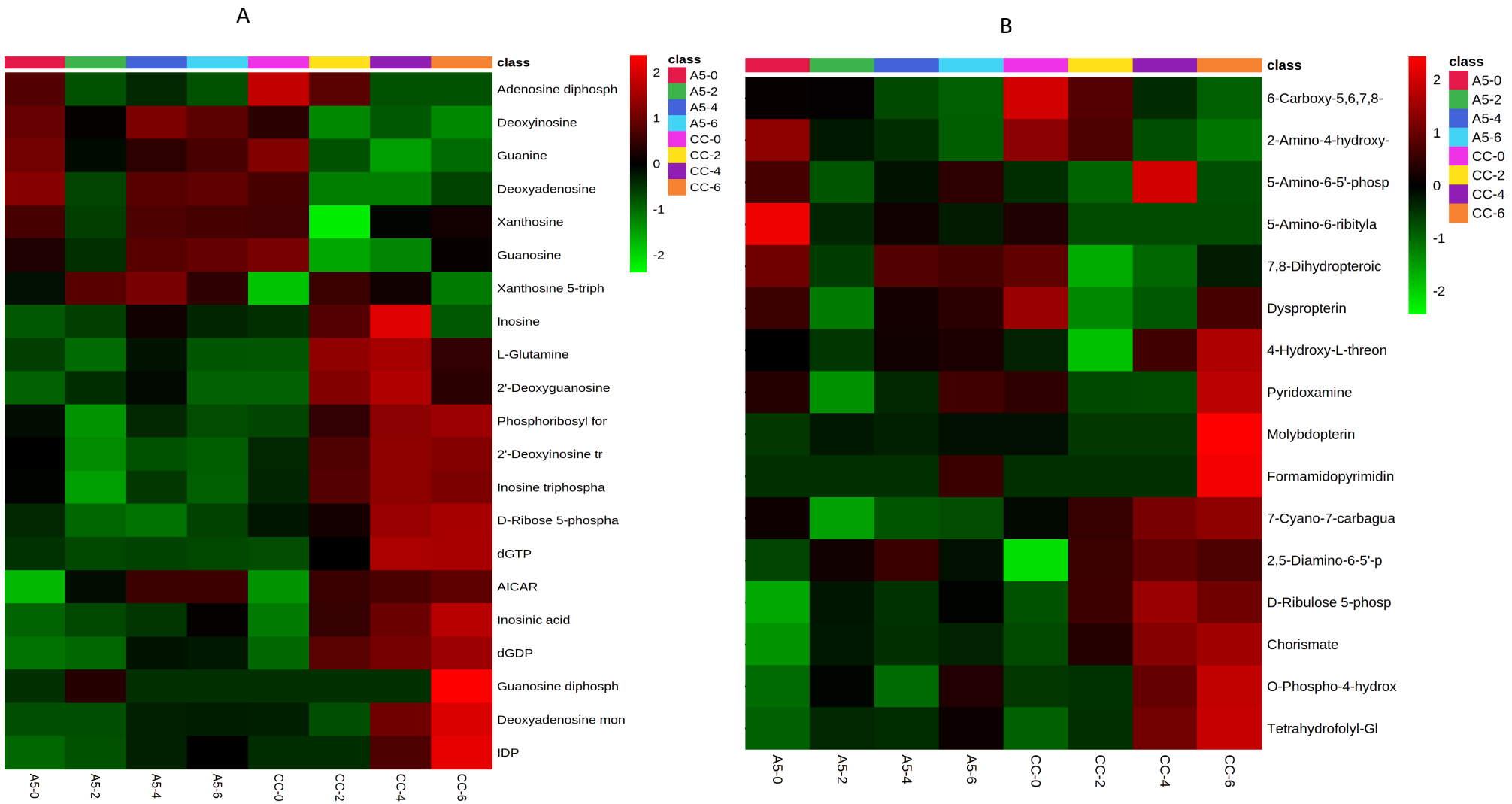


Figure 6. 18: Metabolite changes of MRSA treated with A5 in comparison to untreated cells

A: Heatmap of all the significantly different metabolite changes happening at different time points in both treated (A5) and non-treated cells (CC) in nucleotide metabolism; B: Heatmap of all the significantly different metabolite changes happening at different time points in both treated (A5) and non-treated cells (CC) in energy metabolism.

The metabolism map with the metabolites of the treated cells suggest that A3 specifically affected the glycogenesis pathway. We can also notice that the downregulation of metabolite takes place at 2h indicated by the heatmap. While in case of A5, we see several other pathways been affected namely fatty acid metabolism, nucleotide metabolism and energy metabolism along with carbohydrate metabolism downregulation at 2h and the presence of common metabolites such as acetic acid and phosphoenolpyruvic acid in the carbohydrate metabolism could be due to the presence of similar compounds.

Observing the overall metabolite changes and the pathways related it can be assumed that A3 massively alters the central metabolism of MRSA. While in case of A5, effects on carbohydrate metabolism including lactic acid production suggest that a switch to anaerobic respiration. Alongside metabolites changes in purine biosynthesis.

6.5. DISCUSSION

In Chapter 5, HB5d/e3(A3) and HB5d/e5 (A5) was demonstrated to have a substantial antimicrobial activity against MRSA, thus it was important to understand their mode of action. Understanding the effects of any antibiotic could be aided if any impact were seen on the ultrastructure of the bacterial cell wall (Alharbi et al., 2017). One method to assess such effects is to use TEM to observe morphological changes in bacterial cells provides useful insights into the mechanism underlying the activity of antibacterial agents (Joung et al., 2014). When bacteria cells were treated with A3 and A5, cell membrane disintegration, cell lysis, and release of cytoplasmic contents were observed (Figure 6.3) and the ultrastructural impact on bacteria cells indicated A3 had stronger antibacterial effects than A5.

Our group has recently applied metabolomic approaches to define the mode of action the pretomanid, a promising anti-tubercular drug currently at clinical phase III. Therefore, metabolomic approaches were employed to define possible modes of action for A3 and A5 on MRSA. We also compared the metabolomic effects of A3 and A5 with those of other antibiotics whose mode of action was more clearly established (Table X). This aimed to link the metabolomic effects on either A3 and A5 with a particular antibiotic and thereby suggest a common mode of action. In the event, the metabolomic approach failed to discriminate between the impacts of the established antibiotics on the MRSA metabolomes, even though their modes of actions were very different (Table X). This could indicate that in these instances the MRSA metabolomes were reflecting a common stress response by the bacteria. However, mapping the significant metabolite changes between the antibiotics and controlled cell, directed towards the potential mode of action for the antibiotics (Figure 6. 6-Figure 6.12).

In this study, we were interested in the metabolic changes brought about by A3 and A5 in the MRSA. We performed extensive characterization and identification of the derived metabolomes, mostly using MetaboAnalyst and the resources of the KEGG database

(https://www.genome.jp/kegg/). On mapping the significant metabolites effected by A3 onto the general metabolic pathway of *S. aureus* we found that glycolysis intermediates —such as phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3-PG)—had highly decreased levels in cells treated with A3. Although the levels of lactate remain unaffected. Interestingly, the levels of pyruvate were increased at 2h and 6h, and the secretion of this metabolite to the medium was also elevated in infected cells (Figure 6. 14). This is suggestive of an upstream block explaining the accumulation of pyruvate. While the nature of this block is unknown, potential targets could be (partial) inhibition of pyruvate kinase and/or the pyruvate dehydrogenase complex.

However, mapping of metabolites effected by A5 onto the general metabolic pathway of *S. aureus* suggested its effects on carbohydrate metabolism as well as different other pathways, including fatty acid metabolism, nucleotide metabolism and energy metabolism. Our results showed that upstream metabolite succinate and downstream metabolite fumarate and malate detected increased levels in TCA cycle, indicating that the TCA pathway might be disrupted and thus in turn effects the energy metabolism at 2h. The presence of lactic acid indicates anaerobic conditions been created at 2h (Krebs, 1937).

Purine biosynthesis contributes to the synthesis of DNA and RNA which, in turn, drives the synthesis of proteins, decreased levels of upstream metabolites such as Ribose-5-phosphate and AICAR indicate disruption in the nucleotide synthesis at 2h. While increased levels of guanine and guanosine indicated alteration in purine nucleoside phosphorylase enzyme at 4h.

Overall evaluation of the related pathways indicated and the significant metabolite changes, it can be assumed that that A3 might alter pyruvate levels in the cells resulting in affecting the overall glycolysis pathway. While A5 results in creating an anaerobic condition thus utilizes energy production through TCA cycle. Confirmation of these modes of action could entail assays of the purified enzymes. However, it is was confirmed that would represent a novel

mode of action not shared by the established antibiotics considered here or the anti-MRSA drugs described in the introduction.

6.6. CONCLUSION

Although it is currently not unequivocally established how A3 and A5 cause the observed MRSA cell death, our metabolomic approach suggested some candidate mechanisms. To what extent these factors are responsible for the main effects or are so-called off-target effects needs further research. Equally, our time course analyses were too crude to define which effect could be happening first and would probably require the use of label substrates and timepoints gathered seconds after application of A3 and A5. However, these could confirm that A3 and A5 represent a new class of anti-MRSA antibiotics.

7

General Discussion

The use of Traditional Chinese medicine (TCM) has been extensively documented over thousands of years (Kuriyama, 1988), as does the use the Indian Ayurveda system which dates back to the 1st millennium BC (Patwardhan, 2005). The Western world knowledge is mainly based on the Greek and Roman culture where medicinal plants were only applied on an empirical basis, without the knowledge on their pharmacological activities or active constituents (Roche, 2006). It was only in the 18th century that Anton von Störck, who investigated poisonous herbs such as aconite and colchicum, and William Withering, who studied foxglove for the treatment of oedema, laid the basis for the rational clinical investigation of medicinal herbs (Roche, 2006). drug discovery from plants started at the beginning of the 19th century, when the German apothecary assistant Friedrich Sertürner succeeded in isolating the analgesic and sleep-inducing agent fromopium which he named morphium (morphine) (Sertuerner, 1817). This triggered the examination of other medicinal herbs, and during the following decades of the 19th century, many bioactive natural products, primarily alkaloids (e.g., quinine, caffeine, nicotine, codeine, atropine, colchicine, cocaine, capsaicin) could be isolated from their natural sources.

However, several challenges contribute to the decline of plant-derived natural products as drug discovery source. The first important challenge is the accessibility of the starting material considering the ecological and legal considerations especially laws dealing with plant access and sharing of benefits, and patentability issues with local governments in the countries of origin. Thereafter, often the available content of natural products is low insufficient for testing for a wide range of biological activities. Besides the accessibility of the plant material, also its quality that is of great importance, available plant material often varies on quality and composition and this can hamper the assessment of its therapeutic claims. Although, a detailed knowledge of the interaction of a drug candidate compound with its molecular target is advantageous for the drug development process, but working on plants makes it challenging to determine the precise molecular mechanism of action of natural products (Corson & Crews, 2007).

In this study we examined TCM's for its antimicrobial properties. This involved the use of several different methodologies were employed in addition to the usual bioactivity guided fractionation strategy. Thus, to overcome the problem of availability of the plant material and low quantity of isolated natural product we tried to establish a method using metabolomics to identify bioactives in a low quantity plant extract. This type of approach has not been used before and can provide novel relevant insights. Additionally, we sought to use computational and metabolomic approaches to understand the interaction between cells and the drug

candidate. These were used to suggest mode of actions that could be used to develop the isolated product into a potential drug lead.

Our first approach was to select TCM's relevant to our study. Our collaborators in China provided us with a list of names randomly selected on the basis of their availability. Although randomly selected plants could have been advantageous in assessing biochemical diversity, but we preferred undergoing a knowledge-based approach, where the TCM's had previous traditional use and some crude scientific data. Based on the ethnopharmacological data, we selected 16 plants and 2 fungal species to screen for antimicrobial components (Chapter 3). As the TCMs were commercially obtained by our collaborators it was essential to authenticate them. We used DNA barcoding approach to authenticate the material where we came across several mislabeling which were confirmed with the difference in their biological activities.

It had been previously reported that although the antimalarial agent artemisinin itself is not active against tuberculosis, conjugation to a mycobacterial-specific siderophore analogue induces significant and selective anti-tuberculosis activity, including activity against multi-and extensively drug-resistant strains of *Mycobacterium tuberculosis* (Miller et al., 2011). This led us to explore *Artemisia annua* as a source of antimycobacterial components, as *A. annua* produces several artemisinin derivatives in its natural pathway (Chapter 4). We took the traditional approach to start pharmacological testing with crude extracts and subsequently isolate and characterize the constituents responsible for the activity of the extract. We selected solvent from non-polar to polar for the crude extraction, crude extracts exhibiting biological activity are subjected to iterative bioactivity-guided fractionation cycles until the respective pure bioactive compounds are identified. Two artemisinin derivatives were isolated with some antimycobacterial activity (dexyartemisinin:0.5 μg mL^{-1} artemisinic acid:0.5 μg mL^{-1}). *A. annua* had not been previously examined for its antimycobacterial properties. However, the problem we faced here were the low insufficient quantity of the compounds to use in mode of action studies. We did attempt to obtain more starting material from a different source but there was a difference in the pharmacological data of the crude extract (Chapter 3). Another problem was that we observed an increased MIC after fractionation, this may be the result of synergistic interactions being isolated in different fractions. We undertook computational modeling to predict a drug target for both the compounds among known six mycobacterial targets, which suggested that MtKasA could be a drug target for artemisinic acid.

The next plant that was worked on was *Dryopteris crassirhizoma* Nakai traditionally used for viral treatments (Chapter 5). Phytochemical studies have shown that *D. crassirhizoma* is rich in triterpenes, flavonoids and phloroglucinols. Based on the anti-MRSA data obtained while

screening the crude extract in chapter 3, we undertook bioassay guided purification to find the key metabolites same as we did for *A. annua*. Unfortunately, even after undergoing successive series and different methodologies of chromatographic separation, we failed to isolate a pure compound to that it could be identified by NMR. However, given the substantial anti-microbial activity, we attempted to identify the targeted key metabolites using UHPLC-MS and tandem mass spectroscopy (MS^2/MS^3) data. Comparison of the data with several literature we identified them as phloroglucinol derivatives norflavaspidic acid AB and flavaspidic acid AB. Although flavaspidic acid AB has been previously reported to have anti-MRSA activity (MICs ranging between 12-20 μg/mL), this was the first time that his has been reported for norflavaspidic acid AB. Thus, it was essential to investigate the mode of action of norflavaspidic acid AB and flavaspidic acid AB. We intended to undergo cytotoxic and antischistosoma assessments as described chapter 4, but these were not undertaken due to time restrain.

Metabolomics approaches are increasingly playing important role in drug discovery. Unlike targeted approaches, nontargeted metabolomics allows the detection and analysis of large numbers of features, providing a fingerprint or 'global profiling' of the sample being studied. In this thesis, this approach was followed to describe microbial pathways disturbed by a drug, after metabolomics analysis by FIE-HRMS. We intended to explore this property of metabolomics in identifying key metabolites responsible for the activity. In chapter 5 we came across several active fractions (MIC<50 μg/mL) of *D. crassirhizoma*, the best (HB fraction) one was chosen for the traditional way to isolate pure compound, while another fraction (HL fraction), due to its availability, and difference in polarity was chosen to identify compounds via metabolomics. One step of column chromatography followed by FIE-HRMS was undertaken. Based on statistical tests done using MetaboAnalyst, searching the databases and previous literature, 3 compounds were identified filixic acid ABP, flavaspidic Acid AB, and dryocrassin ABBA. This approach was taken mainly to focuses on the fact that the bioactivity of a plant extract may be the result of synergistic interactions of several components, and in this case bioactivity guided fractionation might fail, as observed in case of *A. annua*. However, more research is needed in this direction in understanding the stability and enhanced bioavailability in the extract.

The use of metabolomics in drug target discovery is considered as central for an effective investigation for new antibiotics against multi-resistant microorganisms. This approach has been previously demonstrated in our group where a new mode of action was determined for anti-mycobacterial pretomanid (Baptista, Fazakerley, et al., 2018). We used the same approach

with some modifications to understand the mode of action of the fraction HB5d/e3 and HB5d/e5 containing different concentrations of norflavaspidic acid AB and flavaspidic acid AB (Chapter 6). Flow infusion ionization electrospray high-resolution mass spectrometry (FIE-HRMS) and biochemical pathway assessment indicated the anti-MRSA activity of HB5d/e3 was strictly linked to the central glycolysis pathway, possibly including the inhibition of pyruvate kinase and/or the pyruvate dehydrogenase complex. While HB5d/e5 showed the effect on variety of metabolic pathways including amino acid biosynthesis, nucleotide biosynthesis, fatty acid biosynthesis and energy. Considering the concentration of norflavaspidic acid AB and flavaspidic acid AB in both the fractions, HB5d/e3 shows higher quantity of norflavaspidic acid AB, while HB5d/e5 shows equal quantity of both compounds. Presence of similar metabolite changes of the central metabolism in the biochemical pathway assessment of both the fractions suggests that the anti-MRSA activity of norflavaspidic acid AB might be selectively affecting the glycolysis pathway to influence the bacterial bioenergetic metabolism.

Glycolysis is an important process of ATP generation via the substrate level of phosphorylation. The breakdown of glucose generates more ATP in oxidative phosphorylation while the same monosaccharides produce only two net ATP in glycolysis (Flamholz, Noor, Bar-Even, Liebermeister, & Milo, 2013). Also, it has been reported that glycolytic enzymes constitute a large fraction of microbial proteomes and lowering their levels (i.e., by using the Entner-Doudoroff Pathway (ED Pathway) as an alternative to glycolytic pathway) could allow cells to increase their growth rate. Thus, there could be a tradeoff between a glycolytic pathway's ATP yield and the growth rate it can support. It should be noted that glycolytic metabolites were increased with A3 treatment. This could be linked to a suppression of bacterial growth. However, this needs to be further investigated to confirm the exact or multiple enzymes effected by the A3.

In summary, against all the challenges faced throughout this project's results and findings was to revisit traditional medicine and using western approaches to of their effective use of as antimicrobials. Our pipeline for the analysis of commercially brought TCM's (Chapter 3) attempts to overcome some challenges usually faced working with plant based natural products and was successfully in certain cases. Given more time and resources, various other aspects of the study could have been improved such as the major drawbacks in this study was the incomplete databases with ethnopharmacological information of medicinal plant, mainly due to language barriers in terms of literature studies and restricted access to Chinese websites. These barriers prevent the ready assessment of compounds that could be therapeutically

effective in humans. Using untargeted metabolomics solely has challenges such as robust and rapid compound identification, more complete metabolome coverage hinder confidence in this approach and its widespread adoption. Thus, undergoing validation of the metabolites and pathways using functional genomics, proteomics and *in-silico* approaches are required.

Future work, needs to purify norflavaspidic acid AB and flavaspidic acid AB to high yields so that cytotoxicity studies and animal studies can be attempted. The latter could incorporate pharmacokinetic studies that are required to consider these as potential drug leads. Of equal importance is the use of genomics and molecular docking so that mode of action studies can be undertaken. These should inform the design and testing of derivative molecules. This is important as neither norflavaspidic acid AB and flavaspidic acid AB conforms to the Lipinski's rule of five which is used to evaluate drug likeness or an orally active drug in humans(Lipinski, Lombardo, Dominy, & Feeney, 2012). This point notwithstanding, this study has demonstrated how scientific approaches can be employed to define the molecular basis of medicinal properties in TCM.

8

Thesis Output

8.1. PUBLICATIONS

- *Bhowmick et al. (2020)*

Bhowmick S, Baptista R, Fazakerley D, Whatley KCL, Hoffmann KF Shen J, Mur LAJ. (2020) The anti-mycobacterial activity of *Artemisia annua* L is based on deoxyartemisinin and artemisinic acid. (In preparation)

Abstract

The development of anti-parasitic artemisinin from *Artemisia annua* L. is an example of how Traditional Chinese Medicine (TCM) may be exploited to meet a recognized need. We systemically investigated *A. annua* for its antimicrobial activity which has not previously reported. In this study, we assessed *A. annua* as a source of bioactive natural products for anti-mycobacterial activity.

Anti-mycobacterial activity-guided purification of the *A. annua* leaf on a silica gel column and structure elucidation using UHPLC-HRMS and NMR resulted in the identification of active compounds. Crude extracts, isolated compounds and artemisinin (Apollo Scientific Ltd) used as positive control were assessed against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), Mycobacterium smegmatis strains by serial micro dilution method (31.25-1000 µg/mL). Anthelmintic activity of isolated compounds and artemisinin against *Schistosoma mansoni* was analyzed by the Roboworm anthelmintic discovery platform. The isolated compounds were tested for synergistic effects against mycobacterium. We also used a molecular docking approach to investigate the interactions between selected anti-mycobacterial compounds and proteins

involved in vital physiological functions in *M. tuberculosis*, namely MtPks13, M*t*PknB, MtPanK, MtKasA, MtInhA and MtDprE1.

Identity of *A. annua* was confirmed by *rbcL* DNA barcoding, bioactive compounds were purified and identified as deoxyartemisinin and artemisinic acid. Artemisinic acid (MIC 250 µg/mL) was more effective in comparison to deoxyartemisinin (MIC 500 µg/mL) and artemisinin (MIC 1000 µg/mL) against *M. smegmatis*. These did not exhibit any anthelmintic activity against *S. mansoni*. The docking score for ligands towards each protein was calculated to estimate the binding free energy, with the best docking score (lowest energy value) indicating the highest predicted ligand/protein affinity. Artemisininc acid. Considering the MIC, artemisinic acid showed docking scores superior to the control inhibiters for MtKasA, and can be a potential nick for further in vitro biological evaluation and anti-TB drug design.

- *Bhowmick et al. (2020)*

Bhowmick S, Baptista R, Han J, Li F, He W, Shen J, Mur LAJ. (2020) Traditional Chinese medicinal herbs: a potential source of anti-microbials (In preparation)

Abstract

Ethnopharmacological relevance: With the increased emergence of antimicrobial resistant bacterial strains, it is important to find new drugs. Identifying drug leads from natural products has proven to be an important strategy in anti-infectious drug research. Natural products have a privileged role in drug discovery due to their intrinsic cell permeability, structural diversity, rich functionality and stereochemistry. They also provide scaffolds for further drug optimisation towards increased potency and selectivity. China has been using herbs in medicine for centuries (Traditional Chinese Medicine) and these are now attracting global interest information regarding their use is often inaccessible to the wider scientific community

Aim of the study: Our project focused on 18 Chinese herbs, traditionally used to treat infections, which were assessed for their anti-microbial properties.

Materials and Methods: Following interrogation of Chinese sources, commercially sourced samples of targeted species were assessed using *rbcL and ITS* barcoding. Extracts with different polarities for selected species were screened for their anti-microbial activity against *E. coli*, S. aureus, methicillin-resistant *S. aureus*, *M. smegmatis*, *P. aeruginosa* and *C. albicans* using the 96-well plate micro-dilution method.

Results and Discussion: DNA barcoding indicated that some herb samples were mislabelled. *In vitro* studies for anti-microbial activity demonstrated that some extracts showed activity against Gram-positive bacteria (*S. aureus*, methicillin-resistant *S. aureus*), *M. smegmatis* and *C. albicans*, while all tested Gram-negative bacteria were resistant to all the extracts. The highest activities were shown by *n*-hexane and ethyl acetate extracts of *Dryopteris crassirhizoma* against *S. aureus* and methicillin-resistant *S. aureus* (MIC= 6.25µg/mL) and n-hexane fraction of *Oldenlandia diffusa* was against *S. aureus* (MIC= 62µg/mL). However, some samples exhibited sample-to-sample variation in activities.

Conclusion: Our studies target new herb sources of anti-microbial activity that can be further characterised by the scientific community. We also show how rigour is required in sample identification if obtained from commercial sources and confirmation of activities from different sample sources is required before robust conclusions of any intrinsic anti-microbial activities can be made.

- *Bhowmick et al. (2020)*

Bhowmick S, Baptista R, Robert E, Bozhilova KS, Mur LAJ. (2020) Robust identification of bioactives using FIE-HRMS and UHPLC-HRMS. (In preparation)

Abstract:

Antimicrobial resistance is a global threat to the health systems and the individual well - being, for example, the methicillin - resistant strain of *Staphylococcus aureus* (MRSA) is known to cause over 11,000 deaths and 80,000 invasive infections every year. New antibiotics and antimicrobials are required but current strategies for their development is failing to keep up with demand. Therefore, researchers are searching for alternative sources for new antimicrobial substances and antibiotics. Eastern medicine and the traditional Chinese medicine have been proven effective against infections and diseases that the Western medicine is still developing cures for. The current research looks into the rhizomes of *Dryopteris crassirhizoma* - an evergreen fern that is known for its anti - inflammatory and antimicrobial properties. DNA barcoding, column chromatography and antimicrobial assays established that the authenticity of the plant and a n - hexane fraction exhibits the highest antimicrobial activity against two strains of *S. aureus* and MRSA. The *n*- hexane fraction underwent fractionation and one-designated - HL - was chosen to be further assessment. UPLC-MS and FIE-HRMS followed by metabolomic comparison of active and non-active sub-fractions of HL coupled with interrogation of databases lead to the tentative identification of some of the bioactives. Three compounds, flavaspidic acid AB, filixic acid ABP and dryocrassin ABBA were targeted as, in least in part, responsible for its antimicrobial activity against *S. aureus* and MRSA. Further purification using preparative-HPLC and tests such as IR, 1H- and 13C -NMR, 2D-NMR and circular dichroism (CD) are required for full structural elucidation. In conclusion the aim of this project was to find a rapid method for the tentative identification of bioactive compounds.

8.2. POSTER PRESENTATION

- *Bhowmick et al. (2017)*

PRIFYSGOL ABERYSTWYTH UNIVERSITY
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Institute of Biological, Environmental and Rural Sciences

Exploiting natural anti-mycobacterial products from the Chinese herb Artemisia annua

中国中医科学院 青蒿素研究中心
Artemisinin Research Center
China Academy of Chinese Medical Sciences

Sumana Bhowmick [1], Rafael Baptista [1], David Fazakerley [1], Jianying Shen [2], Luis A. J. Mur [1]

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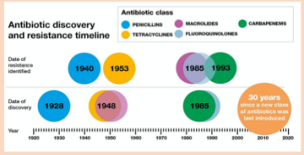
Introduction

Tuberculosis is the leading cause of death from an infectious disease; causing 1.5 million deaths in 2015. With the increased drug resistance, new drugs were developed.

The search of new natural products against ***Mycobacterium tuberculosis*** has assumed some prominence. Natural products are exciting interests as new antimycobacterials due to their intrinsic cell permeability, structural diversity, rich functionality and stereochemistry providing unique scaffolds for further drug optimisation towards increased potency and selectivity.

Traditional Chinese Medicine (TCM) uses herbs as medicine for centuries. Though it is still considered as pseudoscience, it is very attractive to the western counties for research. The western knowledge have given ways to effectively use these herbs and study of compounds responsible for their effectivity.

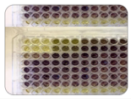
Artemisia annua, commonly called sweet wormwood, is native to temperate Asia. **Artemsinin** was isolated **Prof You-You Tu** in the 1960's for which she was awarded the Noble Prize in Physiology and Medicine in 2015. It was isolated from the aerial part of *Artemisia annua* and since has become a first line antimalarial drug.



Method Pipeline

Plant material
The aerial part plant was dried and powdered

Extraction
The plant was dissolved in different solvents in order to extracts constituents based on polarity



Screening
The crude extracts were screened against different bacteria

Purification
The extracts were then purified using silica gel column chromatography into pure compounds

Characterization
Exactive™ Plus Orbitrap Mass Spectrometer was used to identify the exact mass of the pure compounds

Characterization
Nuclear magnetic resonance (NMR) was used to determine the structure

Figure 1: The identity of the active compounds

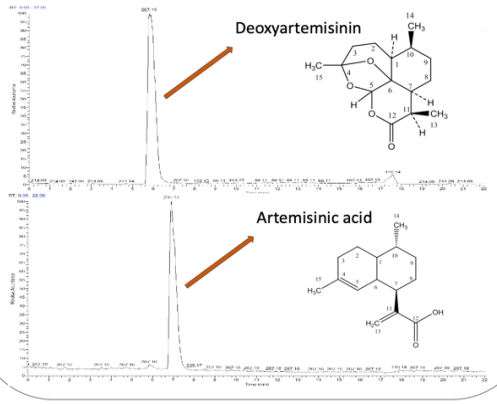
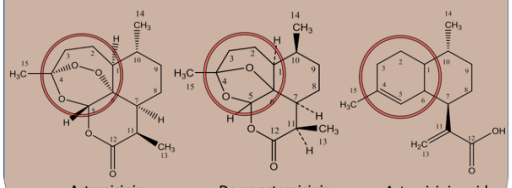


Figure 2: Structural Differences



Antimicrobial activities

Organism	Deoxyartemisinin [mg/ml]	Artemisinic acid [mg/ml]	Artimesinin [mg/ml]
E. coli	0.5	NT	≥1
P. aeruginosa	0.5	NT	≥1
S. aureus	0.5	NT	≥1
M. smegmatis	0.5	0.5	≥1
S. aureus MRSA	0.5	NT	≥1

NT = not tested

Conclusion

- Artemisinin exhibits very poor antimicrobial properties. However, *Artemisia annua* has never been systematically assessed for anti-microbial activity.
- We undertook as extensive series of extractions and based on bacterial killing assays of pathogens.
- Two compounds were shown to have anti-microbial activity (Figure 1):
  - **Artemisinic acid**
  - **Deoxyartemisinin**
- Neither had a endoperoxide bridge suggesting an novel anti-microbial mechanism (Figure 2)
- Automated screens against *Schistosoma mansoni* using the Roboworm platform showed that deoxyartemisin has no anti-parasite activity (not shown)

Barrett Centre for Helminth Control

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- *Bhowmick et al. (2018)*

Defining antimicrobial compounds in traditional Chinese medicinal herbs

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Abstract:
Traditional Chinese Medicines (TCM) ; derived following generations of practice represent an excellent source of potential drug leads; but often these are poorly characterised. We are Chinese-UK collaborative team that is aiming to define the biochemical basis of anti-microbial and anti-parasite TCM, focusing on defining mode-of-action (MoA) in order to provide a firm basis for drug development. We have developed a robust discovery pipeline that 1) catalogues and validates (through sequencing) targeted TCML 2) uses bioactivity based assays to identify the biochemical basis of the TCM and 3) post/genomic approaches to define the MoA.

Keywords: Traditional Chinese medicine, plant extracts, antimicrobial, natural products.

Introduction:
The traditional use of herbs as medicine in China dates back more than 3000 years. TCM represent an important corpus of "folk-medicine". The Chinese *Materia Medica* describes thousands of medicinal substances—primarily plants — which are often combined in formulas and given as teas, capsules, liquid extracts, granules, or powders. TCM however, are often relatively uncharacterised using modern pharmacological methods. As a result drug leads that could be developed to mean globally important health issues; cancer, diabetes, Alzheimer's and infectious disease, are missed. Further, innovation in '-omics' technologies, chemical biology and genetics with the knowledge of TCM will help in the discovery of new drugs and new drug targets. **In this current work we describe how a Chinese- UK collaborative team has developed a pipeline (Fig. 1) for anti-microbial and parasite TCM drug discovery.**

Figure1: TCM bioactives discovery pipeline

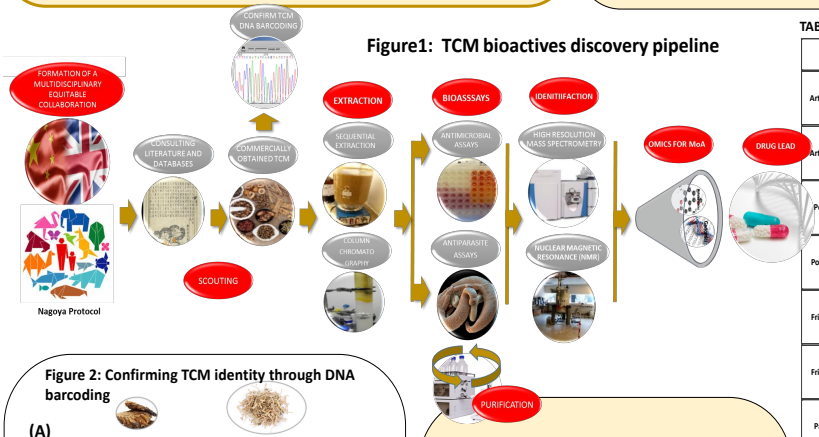
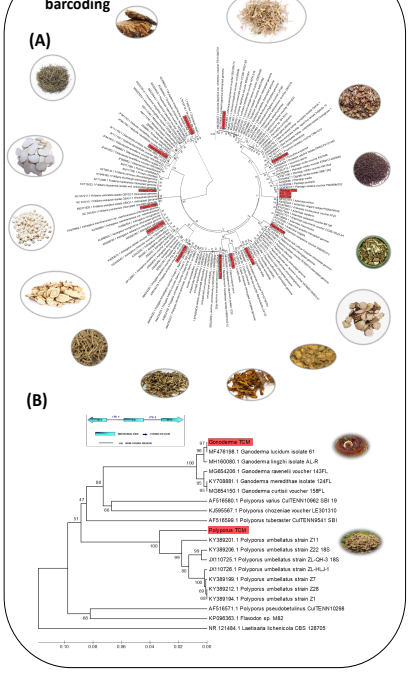


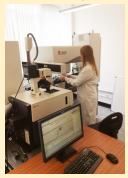
TABLE 1 : Minimum inhibitory concentrations for targeted TCM

Plants	Solvents	E. coli	P. aeruginosa	S. aureus	MRSA	M. smegmatis	C. albicans
Artemisia TCM1	n-Hexane	≥500	≥500	500	500	500	500
	DCM	≥500	≥500	≥500	≥500	≥500	500
	EtOAc	≥500	≥500	≥500	≥500	≥500	500
	MeOH	≥500	≥500	≥500	≥500	≥500	500
Artemisia TCM2	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	≥500	≥500	≥500	500
	EtOAc	≥500	≥500	≥500	≥500	≥500	500
	MeOH	≥500	≥500	≥500	≥500	≥500	500
Polygala TCM	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	≥500	≥500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Polyporus TCM	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	≥500	≥500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Fritillaria TCM1	n-Hexane	≥500	≥500	≥500	≥500	250	≥500
	DCM	≥500	≥500	≥500	≥500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Fritillaria TCM2	n-Hexane	≥500	≥500	≥500	≥500	500	≥500
	DCM	≥500	≥500	≥500	≥500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Paeonia TCM	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	≥500	≥500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	500
Ganoderma TCM	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	250	≥500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Lonicera TCM	n-Hexane	≥500	≥500	500	≥500	500	≥500
	DCM	≥500	≥500	500	≥500	≥500	≥500
	EtOAc	≥500	≥500	250	250	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Forsythia TCM	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	500	500	≥500	≥500
	EtOAc	≥500	≥500	500	500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Oldenlandia TCM	n-Hexane	≥500	≥500	62.5	125	500	≥500
	DCM	≥500	≥500	≥500	500	≥500	≥500
	EtOAc	≥500	≥500	250	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Astragalus TCM	n-Hexane	≥500	≥500	≥500	250	500	≥500
	DCM	≥500	≥500	≥500	500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	500	≥500
Coptis TCM	n-Hexane	≥500	≥500	≥500	≥500	250	≥500
	DCM	≥500	≥500	250	500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	125	500	≥500	250
Corydalis TCM	n-Hexane	≥500	≥500	≥500	≥500	≥500	≥500
	DCM	≥500	≥500	500	500	≥500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Plantago TCM	n-Hexane	≥500	≥500	≥500	≥500	500	≥500
	DCM	≥500	≥500	500	≥500	500	≥500
	EtOAc	≥500	≥500	≥500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Menispermum TCM	n-Hexane	≥500	≥500	250	250	250	≥500
	DCM	≥500	≥500	500	250	250	≥500
	EtOAc	≥500	≥500	500	≥500	≥500	≥500
	MeOH	≥500	≥500	≥500	≥500	≥500	≥500
Dryopteris TCM	n-Hexane	≥500	≥500	15	15	500	≥500
	DCM	≥500	≥500	125	62.5	500	≥500
	EtOAc	≥500	≥500	31.25	15	500	≥500
	MeOH	≥500	≥500	≥500	500	≥500	≥500
Antibiotics		1.6	3.13	3.13	3.13	1.14	

Figure 2: Confirming TCM identity through DNA barcoding



Our collaborative team has examined the Chinese literature to target TCM which have been used to treat infectious disease or wounds.
The TCM is sources from Chinese sources and it identity is confirmed by "DNA barcoding" based on the *rbcS* gene for plants (Fig. 2A) and the rRNA internal spacer region (ITS) for fungi (Fig 2B) . Our TCM products are indicated in red.
Solvent extracted samples are tested for activity in high throughput screens against clinical relevant strains of
E.coli, Pseudomonas aeruginosa, **Multi-drug resistant** ***Staphylococcus aureus*** **(MRSA),** ***Mycobacterium smegatis.*** (Table 1)
The most highly active TCM are indicated in red.
Also against the parasite, ***Schistosoma mansoni ,*** a water-borne parasite of humans, using the Roboworm platform (left).
The active compounds are purified through sequential Rounds of semi-preparative HPLC and identified by NMR and high resolution mass spectroscopy.
Mode of action studies involve Genetic assessment of derived TCM Resistant bacterial mutants , proteomics and metabolomics as we have recently demonstrated or pretomanid (Baptista *et al.*, 2018) ; as well as computer modeling of target interactions



Acknowledgements: SB is funded through Aberystwyth University PhD Studentship. RB acknowledges the Life Sciences Research Network Wales for its PhD Studentship.

- Baptista R, Fazakerley DM, Beckmann M, Baillie L, Mur LAJ. Untargeted metabolomics reveals a new mode of action of pretomanid (PA-824). Sci Rep-Uk. 2018 2018/03/23;8(1):5084.

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8.3. NON-THESIS RELATED OUTPUT

- *Baptista et al. (2018)*

Baptista R, Bhowmick S, Nash RJ, Baillie L and Mur LAJ (2018). Target discovery focused approaches to overcome bottlenecks in the exploitation of antimycobacterial natural products. *Future Medicinal Chemistry* 10(7), 811-822.

Abstract:

Tuberculosis is a major global health hazard. The search for new antimycobacterials has focused on such as screening combinational chemistry libraries or designing chemicals to target predefined pockets of essential bacterial proteins. The relative ineffectiveness of these has led to a reappraisal of natural products for new antimycobacterial drug leads. However, progress has been limited, we suggest through a failure in many cases to define the drug target and optimize the hits using this information. We highlight methods of target discovery needed to develop a drug into a candidate for clinical trials. We incorporate these into suggested analysis pipelines which could inform the research strategies to accelerate the development of new drug leads from natural products.

- *Crusco et.al. (2019)*

Crusco, A.; Baptista, R.; Bhowmick, S.; Beckmann, M.; Mur, L. A. J.; Westwell, A. D.; Hoffmann, K. F. The Anti-Mycobacterial Activity of a Diterpenoid- Like Molecule Operates through Nitrogen and Amino Acid Starvation. Front.Microbiol. 2019. https://doi.org/10.3389/fmicb.2019.01444.

Abstract:

A library of 14 minimally cytotoxic diterpenoid-like compounds (CC50 > 70 μM on HepG2 human liver cells) was screened against *Mycobacterium smegmatis*, *Staphylococcus aureus*,

and *Escherichia coli* to determine antimicrobial activity. Some compounds with a phenethyl alcohol (PE) core substituted with a β-cyclocitral derivative demonstrated anti-mycobacterial activity, with the most active being compound 1 (MIC = 23.4 mg/L, IC50 = 0.6 mg/L). Lower activity was exhibited against *S. aureus*, while no activity was displayed against *E. coli*. Low cytotoxicity was re-confirmed on HepG2 cells and additionally on RAW 264.7 murine macrophages (SI for both cell lines > 38). The sub-lethal (IC50 at 6 h) effect of compound 1 on *M. smegmatis* was examined through untargeted metabolomics and compared to untreated bacteria and bacteria treated with sub-lethal (IC50 at 6 h) concentrations of the antituberculosis drugs ethambutol, isoniazid, kanamycin, and streptomycin. The study revealed that compound 1 acts differently from the reference antibiotics and that it significantly affects amino acid, nitrogen, nucleotides and folate-dependent one-carbon metabolism of *M. smegmatis*, giving some insights about the mode of action of this molecule. A future medicinal chemistry optimization of this new anti-mycobacterial core could lead to more potent molecules.

- *Baptista et al. (2019)*

Baptista R, Fazakerley DM, Bhowmick S, Piński A, Pires D, Anes E, Catalão MJ, Macedo AR, Lloyd DG, Taylor EJ, Whatley KCL, Hoffmann KF, Nash RJ, Fernandez-Fuentes N, Beckmann M, Baillie L, Mur LAJ. (2018) Lupulone targets mycobacterial cell membrane homeostasis through interaction within the MlaDc subunit of the virulence factor mammalian cell entry. (In preparation)

Abstract:

Tuberculosis represents a major global threat due to the prevalence of both drug-resistant and multi-drug resistant TB strains. This requires the development of novel drug classes. Hop *α*-acid lupulone has established activity against mycobacterial species and we here define it molecular target representing a novel mode of action. Subsequent to confirming lupulone's

activity against *Mycobacterium* species including *M. tuberculosis*, we employed the model species *M. smegmatis*. Thus, we derived five spontaneously mutant lupulone-resistant *M. smegmatis* strains whose genomes were sequenced. Three mutations where observed that were consistently present in all strains: a hypothetical protein, an efflux pump and a component of the virulence factor mammalian cell entry (MCE) complex, MlaD subunit C. Only over expression with MlaDc in *M. smegmatis* could increase the minimum inhibitory concentration (MIC) against lupulone. Protein modelling revealed lupulone interaction sites that were abolished in mutated MlaDc alleles. MlaD is predicted to transport phospholipids to the bacterial inner membrane and metabolomic and lipidomic approaches indicated that lupulone imposed changes of *M. smegmatis* metabolism that were distinctive to any other tested antibiotics (linezolid, isoniazid, kanamycin, ethionamide, rifampicin and streptomycin) and perturbed phospholipid accumulations patterns. Thus, we have identified a new druggable target for anti-mycobacterial activity which mediates bacterial membrane processing.

- *Molehin et al. (2018)*

Molehin O, Baptista R, Bhowmick S, Mur LAJ, Oloyede O. (2018) Clerodendrum volubile (P.) Beauv, a case study of a traditional West African medicinal plant with important pharmacological activities.(In Preparation)

Abstract:

Aim of the study: In this review, we provide an overview of *C. volubile* as an exemplar of a West African traditional medicinal plant highlighting its potential significance but equally the remaining challenges before it, and other medicinal plants, can be exploited. Materials and Methods: A review of the literature (ethnobotanical books and publications (published/unpublished resources) reporting traditional plant use in West Africa) was undertaken related to specific medicinal use for *Clerodendrum volubile.* An in-depth analysis

of earlier studies was undertaken and future considerations are highlighted. Results: Scientific studies on extracts of plants have suggested antioxidant, anti-inflammatory, antidiabetic, antihypertensive, hypoglycemic and hypolipidemic properties of the extracts of *C. volubile*. Isolated bioactives include protocatechuic acid, iridoid glycosides, pectolinagarin, biochanin which exhibit antidiabetic, anti-inflammatory, anti-carcinogenic and hepatoprotective activities. Therefore, whilst a few ethnobotanical claims for *C. volubile* have been validated by pharmacological studies, much more work is still required. Conclusions: As this situation is not unique to *C. volubile*, we suggest how West African medicinal plants could scientifically developed into an important international resource for potential drug leads.

- *Baptista et al. (2020)*

Baptista R Bhowmick S, Mur LAJ. (2020) In silico approach to suggest molecular targets of selected anti-mycobacterial natural products (In preparation)

Abstract:

Tuberculosis (TB) is a major global threat mostly due to the development of antibiotic resistant forms of *Mycobacterium tuberculosis*, the causal agent of the disease. Driven by the pressing need for new anti-mycobacterial agents, several natural products (NPs) have been assessed for their in vitro against *M. tuberculosis*. However, the utility of any NP as a drug lead will be augmented if the anti-mycobacterial target(s) is unknown. To accelerate the develop of new NP-based drug lead, we used a molecular docking approach to predict the interactions of 53 anti-mycobacterial NPs against known 'druggable' mycobacterial targets ClpP1P2, DprE1, InhA, KasA, PanK, PknB and Pks13. The docking scores / binding free energies- were predicted using AutoDock Vina were calculated along with physicochemical and structural properties of the NPs, using PaDEL descriptors. The specific interactions of the bisbenzylisoquinoline alkaloids 2-nortiliacorinine, tiliacorine and 13'-bromotiliacorinine

against the targets PknB and DprE1 (-11.4, -10.9 and -9.8 kcal.mol-1; -12.7, -10.9 and -10.3 kcal.mol-1, respectively) were further analysed as well as the interactions of the lignan α-cubebin and Pks13 (-11.0 kcal.mol-1), due to their significantly superior docking scores compared to the respective control inhibitors. We suggest that our approach offers a means to concentrate research aiming to optimise NP and Hence, this study presents new anti-mycobacterial candidates and its targets for potential drug optimisation.

- *Cao et al. (2020)*

Cao D, Wang M, Dai J, Li Y, Bhowmick S, Mur LAJ, Wei Y, Sun Z. (2020) Mining the secondary metabolites from endotype fungi of *Huperzia serrata* for medicinal activities (In preparation)

Abstract:

Huperzia serrata is a genus including ca. 100 species in the Huperziaceae family. In Traditional Chinese Medicine (TCM) decoctions that include *H serrata*. stems have been used as a styptic, antispasmodic and analgesic and for the cure of better blood circulation, contusion, strain, swelling and schizophrenia. To date, six species in the *Huperzia* genus have been chemically investigated, in order to alleviate the pressure on plant resources, more than 300 endophytic fungi have been isolated from *Huperzia serrata*.

This review will consider key features of *Huperzia Serrata,* including its botanical characterization, medicinal resources, traditional uses, phytochemistry, pharmacological research and secondary metabolites of endophytic fungi. Based on this we will explore its future therapeutic and scientific potentials.

- *Cao et al. (2020)*

Cao D, Gao J, Yao K, Zhang Y, Bhowmick S, Nash R,Mur LAJ, Wei Y, Sun Z. (2020) Phlegmarine alkaloid as an intermediate in biosynthesis pathway of *Huperzia serrata*- derived Huperzine A: a metabolomic analysis using UHPLC and FIE-HRMS (In preparation)

Abstract:

Huperzine A (Hup. A) is known as a promising drug candidate for the treatment of Alzheimer's disease (AD). It can be extracted from the natural plant *Huperzia serrata* (*H. serrata*), but rarity of the source cannot meet the needs of clinical therapy. Therefore, researchers have focused their attention on biosynthesis of Hup. A by using phlegmarine alkaloids as the precursors, but the pathway of synthesis has not been well established. In this study, we used metabolomics to link phlegmarine alkaloids to Hup. A in *H. serrata* and employed UHPLC-FIE-HRMS to explore the profiles of metabolites in *H. serrata* collected from different geographical regions. The identified major sources of variation included Hup. A, Hup. A-derivatives and phlegmarine alkaloids. It was found that the contents of Hup. A, Hup. A-derivatives and phlegmarine alkaloids were not all the same in samples from different sources. There was a significant positive correlation between the content of Hup. A, Hup. A-derivatives, and phlegmarine alkaloids Huperzine N/L, Huperzine K and 6-hydroxy-huperzine in particular. These results suggest that an association would be expected if these alkaloids were part of the proposed biosynthesis pathway of Hup. A. This is the first study reporting the relationship between phlegmarine alkaloids and Hup. A in *H. serrata* and may provide evidence-based clues for the development and quality control of *H. serrata* from different natural resources.

- *Cao et al. (2020)*

Cao D, Wang M, Zhang Y, Liu K, Bhowmick S, Mur LAJ, Wei Y, Sun Z. (2020) Efficient enrichment of total alkaloid with higher anti-cholinesterase and antioxidant activities from *Huperzia serrata* by microporous adsorption resins (In preparation)

Abstract:

For the full development and utilization of lycopodium alkaloids from *Huperzia serrata* to treat Alzheimer's Disease, this work was intended to establish an efficient rapid and cost-effective method of separation and purification of these alkaloids through macroporous resin column chromatography. The adsorption and desorption characteristics of total alkaloids on ten macroporous resins were studied first, AB-8 resin was selected as the best adsorbent and can be best described by the Langmuir isotherm model and pseudo-second-order kinetics model. The optimum enrichment conditions were as follows: for adsorption, the concentration, flow rate and volume of the sample were 4.30 mg/mL, 2 BV/h and 10 BV, respectively. For desorption, the alkaloids-loaded AB-8 resin column was desorbed by 7 BV of 80% ethanol at a rate of 1.5 BV/h. After one-step purification, the total alkaloids content had 10.14-fold higher compared to crude extracts with 82.56% recovery yield. Furthermore, alkaloids-enriched extracts exhibited obviously higher antioxidant activity on 2,2-diphenyl-1-picrylhydrazyl radical and xanthine oxidase, and anti-cholinesterase activity (with IC_{50} values of 78.21, 165.43 and 165.28 μg/mL, respectively) in vitro, which also significantly reduced the NO production and iNOS activity in lipopolysaccharide induced N9 cell line. Those results indicated that the AB-8 macroporous resin column chromatography established in this work was a promising method for the industrial-scale purification of total alkaloids from *H. serrata*.

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